NUCLEIC-ACID THERAPEUTICS: BASIC PRINCIPLES AND RECENT APPLICATIONS

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The sequencing of the human genome and the elucidation of many molecular pathways that are important in disease have provided unprecedented opportunities for the development of new therapeutics. The types of molecule in development are increasingly varied, and include antisense oligonucleotides and ribozymes. Antisense technology and catalytic nucleic-acid enzymes are important tools for blocking the expression of abnormal genes. One FDA-approved antisense drug is already in the clinic for the treatment of cytomegalovirus retinitis, and other nucleic-acid therapies are undergoing clinical trials. This article reviews different strategies for modulating gene expression, and discusses the successes and problems that are associated with this type of therapy.

EXOGENOUS NUCLEIC ACIDS
In this context, synthetic oligonucleotides of varying chemistry (typically 16–25 nucleotides), which are introduced into cells by various means, or simply (although inefficiently) by concentration-driven endocytosis.

ANTISENSE
Reverse complement of any DNA or RNA sequence.

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With their promise of high specificity and low toxicity, many believe that gene-targeted therapies will lead to a revolution in cancer therapeutics[1]. Numerous gene-therapy strategies are under development, some of which use nucleic-acid-based molecules to inhibit gene expression at either the transcriptional or post-transcriptional level[2]. This strategy clearly has other potential applications, including in cardiovascular[3,4], inflammatory[5,6] and infectious diseases[7–10], as well as organ transplantation[11].

Although conceptually elegant, the prospect of using nucleic-acid molecules for treating human malignancies and other diseases remains tantalizing, but uncertain[12]. The main cause of this uncertainty is the apparent randomness with which these materials modulate the expression of their intended targets. It is a widely held view that molecule delivery, and selection of which messenger RNA sequence to physically target, are core stumbling blocks that hold up progress in the field. In this review, we recapitulate the development of nucleic-acid drugs for modulating gene expression, discuss newer strategies for solving the problems alluded to above, and detail attempts at using these molecules therapeutically. In so doing, we hope to both educate the reader who is unfamiliar with this literature, and convince those who are sceptical that this remains a viable approach to 'on demand' manipulation of gene expression.

Modulating gene expression

The notion that gene expression could be modified through the use of EXOGENOUS NUCLEIC ACIDS derives from studies by Paterson *et al.*[13], who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell-free system in 1977. The following year, Zamecnik and Stephenson[14] showed that a short (13-mer) DNA oligodeoxynucleotide that was ANTISENSE to the Rous sarcoma virus could inhibit viral replication in culture. On the basis of this work, Zamecnik and Stephenson are widely credited for having first suggested the therapeutic utility of antisense nucleic acids. In the mid 1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was shown[15,16]. These observations were particularly important, because they lent credibility to the belief that 'antisense' was more than just a laboratory phenomenon, and encouraged belief in the hypothesis that reverse-complementary antisense nucleic acids could be used in living cells to manipulate gene expression. These seminal papers, and the thousands that have followed, have stimulated the development of technologies that use nucleic acids to manipulate gene expression. As will be discussed below, virtually all of the available methods rely on some type of nucleotide-sequence recognition for targeting

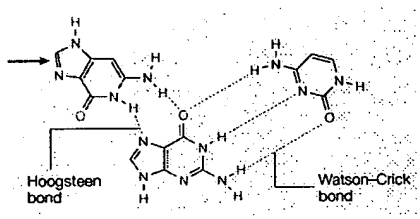

Figure 1 | **Triple-helix formation at the nucleotide level.** Shows the formation of Watson–Crick (red) and Hoogsteen bonds (black) between duplex pairs and the third strand (the arrow points to a single base of the third strand). Blue, guanine residue (purine); pink, cytosine residue (pyrimidine).

specificity, but differ as to where and how they perturb the flow of genetic information.

Strategies for modulating gene expression can be thought of as being either 'anti-gene' or anti-mRNA (see below; reviewed in REF. 2). Anti-gene strategies focus primarily on gene targeting by homologous recombination[17,18], or by TRIPLE-HELIX-FORMING OLIGODEOXYNUCLEOTIDES (TFOs)[19]. As homologous recombination involves vector technology and — at least at the present time — is much too inefficient for clinical use, it will not be considered further in this discussion. TFOs bind in the MAJOR GROOVE of duplex DNA in a sequence-specific manner[20]. Gene targeting with these molecules is constrained by the fact that TFOs require runs of purines on one strand and pyrimidines on the other (~10–30 nucleotides (nts) in length) for stable hybridization. The TFO can be composed of either polypurine or polypyrimidine tracts, but hybridization always occurs on the purine strand of the duplex through the formation of HOOGSTEEN BONDS (FIG. 1).

Successful use of this strategy for blocking transcription and inducing specific mutations, both *in vitro* and *in vivo*, has been reported (reviewed in REF. 20). Although the frequency of such events is typically <1%, Glazer and co-workers[21] have reported a system in which desired mutations can be induced in ~50% of cells, indicating that genuine clinical utility might be possible. This general approach has also been used for inducing mutations that can actually repair a gene that has been made defective by inherited or acquired point mutation. Work to support this concept using chimeric DNA–RNA oligonucleotides has also been reported, but again, the frequency of such repairs, in most cases, has been far too low to be of clinical use at this time[22].

Short, double-stranded (ds)DNA decoy molecules have also been used to disrupt gene expression at the level of transcription[23]. These oligodeoxynucleotides are designed to compete for transcription-factor complexes, with the ultimate goal of attracting them away from the promoter that they would ordinarily activate. For many technical reasons, including limited gene accessibility in the NUCLEOSOME structure, the clinical application of these methods has not progressed at a rapid rate. An alternative approach, using polyamides, or LEXITROPSINS, has been described by Dervan and colleagues[24–26]. These small molecules have the ability to diffuse into the nucleus, where they can contact dsDNA in the minor groove, thereby impeding transcription by preventing unwinding of the duplex, or by preventing the binding of transcription-factor complexes to the gene promoter. DNA accessibility, and maintaining the appropriate 'register' of the polyamides for the desired sequence recognition, are problems with this method that remain to be solved[27].

A larger body of work has focused on destabilizing mRNA. This approach, although less favourable than anti-gene strategies from a stoichiometric point of view, is nonetheless attractive, because mRNA, unlike the DNA of a given gene, is — theoretically— accessible to attack while being transcribed, transported from the nucleus or translated. Two nucleic-acid-based strategies have emerged for blocking translation. One strategy uses oligoribonucleotides. Similar to the strategy of the DNA decoys, the RNA decoys are designed to provide alternate, competing binding sites for proteins that act as translational activators or mRNA-stabilizing elements[28,29]. By attracting away the desired protein, the decoy can prevent translation, or induce instability and, ultimately, destruction of the mRNA. Recent studies on human α-globin mRNA are of interest in this regard. Stability determinants for this mRNA species have been defined in sufficient detail so that it can be used as a model system for testing the hypothesis that altering mRNA stability with decoys will be a useful form of therapy[29–31].

The other strategy for destabilizing mRNA is the more widely applied antisense strategy, which uses RIBOZYMES, DNAzymes, antisense RNA or antisense DNA (ODN). The antisense approach to modulating gene expression has been the subject of numerous authoritative reviews, and will not be discussed in great detail here[2,32,33]. Simply stated, delivering a reverse-complementary — that is, 'antisense' — nucleic acid into a cell in which the gene of interest is expressed should lead to hybridization between the antisense sequence and the mRNA of the targeted gene. Stable mRNA–antisense duplexes can interfere with the splicing of heteronuclear RNA into mature mRNA[34,35], block translation of completed message[36,37] and — depending on the chemical composition of the antisense molecule —lead to the destruction of the mRNA by binding of endogenous nucleases, such as RNaseH[38,39], or by intrinsic enzymatic activity engineered into the sequence, as is the case with ribozymes[40,41] and DNAzymes[42,43] (FIG. 2).

Nucleic acids with catalytic activity

Ribozymes and DNAzymes bind to substrate RNA through Watson–Crick base pairing, which offers sequence-specific cleavage of transcripts. At least six classes of ribozyme have been described. Two ribozymes, the 'hammerhead' ribozyme and the 'hairpin' ribozyme, have been extensively studied owing to their small size and rapid kinetics[44,45]. The catalytic motif is surrounded by flanking sequence that is responsible for 'guiding' the ribozyme to its mRNA target and giving stability to the structure. With the hammerhead ribozyme, cleavage is dependent on divalent cations, such as magnesium, and can occur after any NUH

TRIPLE-HELIX-FORMING OLIGODEOXYNUCLEOTIDE (TFO). A synthetic, single-stranded oligodeoxynucleotide, which, through Hoogsten-bond formation, hybridizes to purine/pyrymidine-rich sequences in double-stranded DNA. Formation of stable triple helices can prevent the unwinding that is necessary for transcription of the targeted region or block the binding of transcription-factor complexes.

MAJOR GROOVE AND MINOR GROOVE
Channels formed by the twisting of two complementary DNA strands around each other to form a double helix. The major groove is ~22 Å wide and the minor groove is ~12 Å wide.

HOOGSTEEN BOND
Triple-helix-forming oligonucleotides hybridize with purine bases that comprise polypurine/polypyrimidine tracks in the DNA. The hydrogen bonds that are formed under these conditions are referred to as Hoogsteen bonds after the individual who first described them. They can form in parallel or antiparallel (reverse-Hoogsteen) orientations.

NUCLEOSOME
A packing unit for DNA within the cell nucleus, which gives the chromatin a 'beads-on-a-string' structure. The 'beads' consist of complexes of nuclear proteins (histones) and DNA, and the 'string' consists of DNA only. A histone octamer forms a core around which the double-stranded DNA helix is wound twice.

LEXITROPSIN
A molecule that extragenetically reads the base sequence of double-stranded DNA.

RIBOZYME
RNA molecule that contains one of a variety of catalytic motifs that cleave RNA to which it hybridizes.

DNAzyme
A DNA molecule that contains a catalytic motif that cleaves RNA to which it hybridizes.

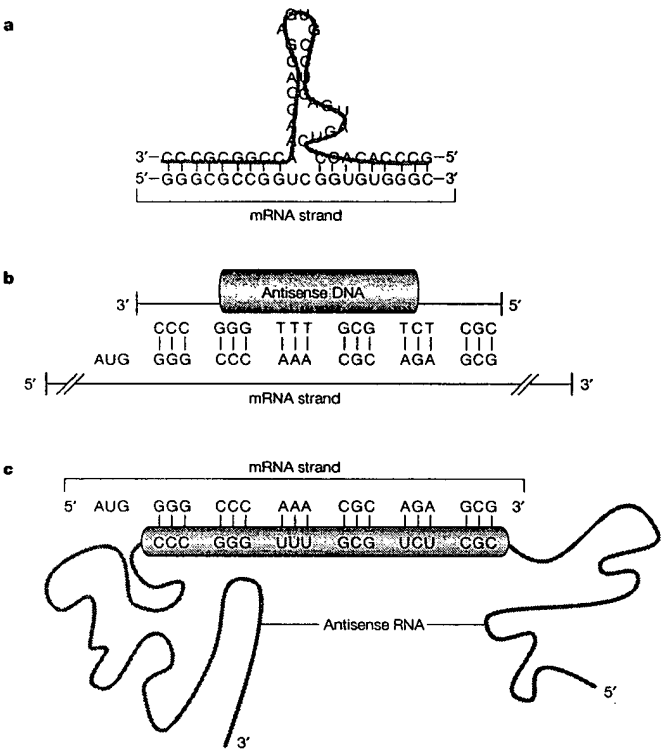

Figure 2 | **Strategies for inhibiting translation.** Diagrammatic representations of **a** | a hammerhead ribozyme (DNAzymes have similar RNA-cleaving capabilities, but the catalytic motif is composed of DNA nucleotides, hence the name); **b** | an antisense oligodeoxynucleotide; and **c** | antisense RNA. Note that targeting specificity is conveyed in each case by Watson–Crick base pairing between complementary sequences. From REF. 2 © (1998) American Society of Hematology, used by permission. mRNA, messenger RNA.

triplet within the target RNA sequence, for which 'N' represents any nucleotide, 'U' represents uracil and 'H' represents adenine, cytosine or uracil[46,47]. If ribozymes are to work effectively as 'enzymes,' they must not only bind substrate RNA but also dissociate from the cleavage product to act on further substrates. Dissociation from the cleavage product might, in fact, be an important rate-limiting step that controls their usefulness[48,49]. Consideration of reaction kinetics indicates that ribozymes might have a theoretical advantage over RNase-H-dependent antisense oligonucleotides, but to the best of our knowledge, this has not been shown consistently *in vivo*. Ribozymes can be expressed from a vector that offers the advantage of continued production of these molecules intracellularly[50,51], a property that — at least until recently — was not possible with antisense DNA[52]. However, it is well known that stable transduction of primary cells *in vivo* has substantial technical problems, which will not be discussed further. Progress has been made recently in synthesizing stable forms of these molecules, so that they might be delivered directly to cells both *in vitro* and *in vivo*[53].

DNAzymes have evolved from the seminal work of Breaker and Joyce[54], who first showed that DNA, as well as RNA molecules, could act enzymatically and cleave a nucleic-acid substrate. Similar to ribozymes, DNAzymes have a catalytic domain that is flanked by two substrate-recognition domains. After binding to their RNA substrate, DNAzymes can cleave sequences that contain purine–pyrimidine junctions. DNAzymes have some theoretical advantages over ribozymes. DNA is more stable than RNA, it is easier to synthesize, and the turnover rates for some of the DNAzymes are reported to be higher than some ribozymes[42]. Nevertheless, constant improvements in both DNAzyme[55] and ribozyme chemistry make this a 'moving target' in terms of which chemistry is better[56]. Although experience with DNAzymes as potential therapeutic agents is limited[43], these molecules might prove worthy in the clinical setting.

RNA interference

A newly developing approach for targeting mRNA is called post-transcriptional gene silencing, or RNA interference (RNAi)[57–58] (FIG. 3). RNAi is the process by which dsRNA targets mRNA for destruction in a sequence-dependent manner. The mechanism of RNAi initially involves processing of long (~500–1,000 nucleotides) dsRNA into 21–25 base-pair (bp) 'trigger' fragments[59] by a member of the RNase-III family of nucleases called DICER[60–62]. When incorporated into a larger, multicomponent nuclease complex named RISC (RNA-induced silencing complex), the processed trigger strands form a 'guide sequence' that targets the RISC to the desired mRNA sequence and promotes its destruction[61]. RNAi has been used successfully for gene silencing in various experimental systems, including petunias, tobacco plants, neurospora, *Caenorhabditis elegans*, insects, planaria, hydra and zebrafish. The use of long dsRNA to silence expression in mammalian cells has been tried, largely without success[63]. More recent reports using short interfering RNA (siRNA; see below) seem to be more promising[64]. It has been suggested that mature, as opposed to embryonic, mammalian cells recognize these long dsRNA sequences as invading pathogens. This triggers a complex host-defence reaction that effectively shuts down all protein synthesis in the cell through an interferon-inducible serine/threonine-kinase enzyme called protein kinase R (PKR). PKR phosphorylates the α-subunit of eukaryotic initiation factor-2 (EIF-2α), which globally inhibits mRNA translation. The long dsRNA also activates 2′,5′-oligoadenylate synthetase, which in turn activates RNase L. RNase L indiscriminately cleaves mRNA. Cell death is the understandable result of these processes. Recently, a number of reports have suggested that siRNA strands — RNA double strands of ~21–22 nucleotides in length — do not trigger this host-defence response, and therefore might be able to silence expression in mammalian somatic cells if appropriately modified to contain 3′-hydroxy and 5′-phosphate groups[66–68]. The universality of this approach, and the types of gene that can be modified using this strategy in mammalian cells, remain unknown at this time.

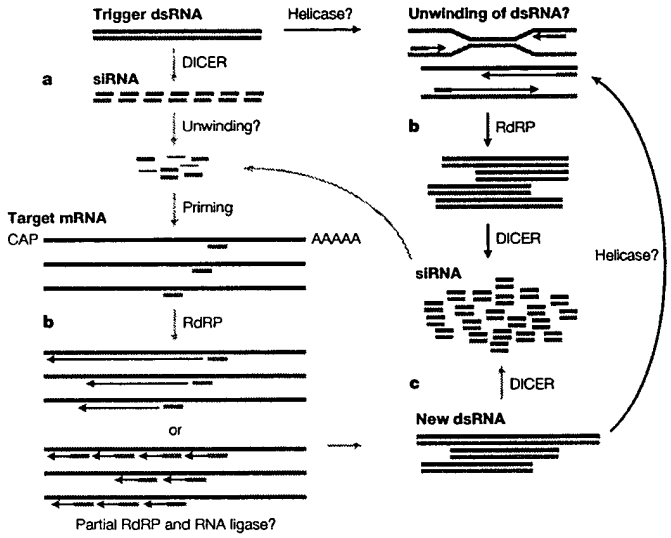

Figure 3 | **Hypothetical RNAi mechanism. a** | In the 'initiation' stage of RNA interference (RNAi), a small amount of trigger double-stranded (ds)RNA is processed into short interfering (si)RNA by an enzyme called DICER (light blue arrow), which is used as an RdRP primer. **b** | The RdRP reaction converts target messenger RNAs into new dsRNAs (next generation of trigger dsRNAs), which are then processed into new siRNAs, establishing a self-sustaining cycle of RNAi 'maintenance' (green arrows). **c** | Replication of 'trigger' or newly synthesized dsRNA by RdRP would amplify the potency of RNAi by further increasing the amount of siRNA, as both sense and antisense strands of trigger dsRNA and siRNA could then be used. However, the *in vivo* significance of this pathway (dark grey arrows) has not yet been established. It also remains unclear if the 'amplification' steps take place in mammalian cells. RdRP, RNA-dependent RNA polymerase; helicase, unwinding enzyme. Redrawn from REF. 57 © (2001), with permission from Elsevier Science.

Altering RNA splicing

Finally, the strategy of manipulating gene expression by altering RNA processing, as opposed to by mRNA destruction, is also worth mentioning, as significant progress seems to have been made in this area. Kole and colleagues developed this approach using a model system based on human thalassaemia[69,70]. Thalassaemias are highly prevalent human blood disorders that are characterized by faulty haemoglobin production and concomitant red-cell destruction that results in anaemia. The genetic mutations that are responsible for these diseases are well characterized, and often involve aberrant splicing. Kole's group showed that treatment of mammalian cells that were stably expressing a human β-globin gene with antisense oligonucleotides that were targeted at the aberrant splice sites blocked the abnormal splicing, thereby allowing the normal splice site to be used. Correction of splicing was oligo-dose dependent and, importantly, led to accumulation of normal human β-globin mRNA and polypeptide in cells[69]. More recently, correction has been accomplished in blood cells derived from thalassaemic patients[71]. This result would clearly have important clinical consequences if such treatment could be made effective at the level of the haematopoietic stem cell. These same workers suggest that this approach might also be useful in the treatment of cancer[72].

Increasing oligonucleotide stability

Initial work with antisense DNA was carried out with unmodified, natural molecules. It soon became clear, however, that native DNA was subject to relatively rapid degradation, primarily through the action of 3′ exonucleases, but also as a result of endonuclease attack. Molecules destined for the clinic, and those used for experimental purposes, are now routinely modified to enhance their stability, as well as the strength of their hybridization with RNA (see REFS 73,74 for further details). Oligonucleotide drugs need to meet certain physical requirements to make them useful. First, they must be able to cross cell membranes and then hybridize with their intended target. The ability of an ODN to form a stable hybrid is a function of its binding affinity and sequence specificity. Binding affinity is a function of the number of hydrogen bonds that are formed between the ODN and the sequence to which it is targeted. This is measured objectively by determining the temperature at which 50% of the double-stranded material is dissociated into single strands, which is known as the melting temperature, or T_m. mRNA-associated proteins and tertiary structure also govern the ability of an ODN to hybridize with its target by physically blocking access to the region that is being targeted by the ODN. Finally, it is also clear that ODNs should exert little in the way of non-sequence-related toxicity[75], and should remain stable in the extracellular and intracellular milieu in which they are situated. Meeting all these requirements in any one molecule has turned out to be a demanding task. Satisfying one criterion is often accomplished at the expense of another. It is also worth noting that the more complex the molecule, the more expensive is its synthesis. In an age of increasing cost consciousness, this too becomes an important design consideration.

First-generation antisense molecules were designed to make the internucleotide linkages — the backbone on which the nucleosides are hung — more resistant to nuclease attack. This was accomplished primarily by replacing one of the non-bridging oxygen atoms in the phosphate group with either a sulphur or a methyl group. The former modification, which is called a phosphorothioate oligodeoxynucleotide, proved highly successful, because these molecules are relatively nuclease resistant, they are charged and therefore water soluble, and they activate RNase H. All of these properties are desirable, and virtually all of the clinical trials done so far have been carried out with this chemistry, although trials using so-called 'second-generation molecules' (mixed backbone/chimeric oligonucleotides) will shortly begin. Second-generation molecules were developed to overcome the disadvantageous properties of the phosphorothioates. A primary strategy that was used was to remove the phosphorothioate linkages to the greatest extent possible. This was often done by flanking a phosphorothioate core with nuclease-resistant nucleosides — often with 2′-*O* sugar modifications — that rendered the molecules more RNA like, and therefore gave tighter binding to the target.

Many chemical modifications to the phosphodiester linkage have been made. Two of the more interesting modifications that are now under development are peptide nucleic acids (PNAs)[76] and MORPHOLINO OLIGODEOXYNUCLEOTIDES (PMOs)[36]. These compounds are essentially nuclease resistant. PNAs represent a more radical approach to the nuclease-resistance problem, as the phosphodiester linkage is completely replaced with a polyamide (peptide) backbone. They both form extremely tight bonds with their RNA targets and probably exert their effects by blocking translation, as neither molecule effectively activates RNase H. Whether it is necessary to preserve the ability of these molecules to activate RNase H is controversial[37], but many workers in the field still believe that molecules with this capability are likely to be more effective, at least in the clinical setting. As these molecules do not move freely across cell membranes, they must be injected or transfected into cells. Finally, PNAs are also sensitive to local ionic concentration and do not hybridize as well under physiological conditions.

MORPHOLINO OLIGODEOXYNUCLEOTIDE (PMO). The base is attached to a morpholino instead of a ribofuranosyl ring, and the backbone is composed of a phosphorodiamidate linkage.

Nucleic-acid drugs in the clinic

Diseases that are characterized by overexpression or inappropriate expression of specific genes, or genes that are expressed by invading microorganisms, are candidates for gene-silencing therapies. For this reason, the earliest clinical trials with these agents have been against human immunodeficiency virus (HIV)77–79 and patients with cancer80. Malignant diseases, in particular, are attractive candidates for this therapeutic approach, if for no other reason than that conventional cancer therapies are highly toxic. As antisense strategies are directed against genes that are aberrantly expressed in diseased cells, it might reasonably be expected that this approach will engender fewer and less serious side effects, as normal cells should not be affected. There were concerns that this might not be the case when preclinical studies on primates with phosphorothioate compounds resulted in the death of some animals. However, investigation of these occurrences showed that they took place after rapid bolus intravenous infusions at concentrations exceeding 5–10 $\mu g\ ml^{-1}$, and that they were probably due to complement activation and vascular collapse[81]. This experience was therefore a useful reminder that, in addition to side effects resulting from the suppression of the targeted gene, side effects related to the chemical backbone of the oligonucleotide should also be anticipated. In the case of phosphorothioates, this problem was easily addressed by infusing material continuously, or slowly, and at lower doses. In actual use in the clinic, phosphorothioates have proved to be remarkably well tolerated (BOX 1). Abnormalities related to the backbone include transient fever, fatigue, nausea and vomiting, mild to moderate thrombocytopaenia and transient prolongation of partial thromboplastin time (PTT; 1.25–1.75 ×), which is fortunately unassociated with any signs of overt clinical bleeding[82–85]. At present, several clinical studies have been carried out using a number of different oligonucleotides. Below, we review some of the more recent clinical studies that have been carried out on patients with malignant, inflammatory, cardiac and infectious diseases (summarized in TABLE 1).

Box 1 | First approved nucleic-acid drug

Vitravene (sodium fomivirsen), an antiviral drug that was developed by ISIS Pharmaceuticals and is marketed by CIBAVision, was approved by European and US regulatory authorities in July 1999 and August 1998, respectively. Vitravene is used to treat an inflammatory viral infection of the eye (retinitis) that is caused by the cytomegalovirus (CMV). CMV often infects immunocompromised patients, and patients with uncontrolled AIDS are particularly at risk. One or both eyes can be affected, and it is not unusual for patients to suffer severe visual impairment or blindness as a result of untreated infections. Treatment of CMV retinitis is problematic, in particular for patients who cannot take, do not respond or become resistant to standard antiviral treatments for CMV infections, such as ganciclovir, foscarnet and cidofovir[145]. Vitravene is an antisense phosphorothioate 21-mer oligonucleotide has a sequence that is complementary to messenger RNA that is transcribed from the main immediate–early transcriptional unit of CMV[145,146].

Targeting apoptosis inhibitors in oncology

BCL2: cancer treatment. Targeting B-cell lymphoma protein 2 (BCL2) is a promising example of triggering apoptosis in tumour cells. BCL2 is an important regulator of programmed cell death, and its overexpression has been implicated in the pathogenesis of some lymphomas[86]. Resistance to chemotherapy, at least *in vitro*, might also be related to *BCL2* overexpression[87,88]. Laboratory studies have shown convincingly that exposing cells to an oligonucleotide targeted to *BCL2* will specifically decrease the amount of targeted mRNA and protein (six–eightfold reduction). For all of these reasons, there is a great deal of interest in targeting *BCL2* for therapeutic purposes[89]. Several clinical trials with a *BCL2*-targeted antisense molecule have been reported, both alone[83,90] and with supplementary chemotherapy[84,91,92]. Studies with the oligonucleotide alone have not shown consistent, strong antitumour responses. The addition of chemotherapy might be helpful in this regard. An issue with several of these studies is lack of correlation of tumour responses with significant effects on BCL2 protein expression. The mechanism of action of the compound is not entirely clear.

Transcription-factor targeting in oncology

c-MYB: bone-marrow purging. The normal homologue of the avian myeloblastosis virus oncogene (v-*myb*) is a proto-oncogene called c-*MYB*. c-*MYB* encodes a protein (MYB), which is a regulator of cell-cycle transition and cellular maturation, primarily in haematopoietic cells, but in other cell types as well. A recently published study was designed to test the hypothesis that an effectively delivered, appropriately targeted ODN might provide a proof of concept about the ability to target a specific mRNA and thereby kill tumour cells selectively[93]. To test this hypothesis, an ODN targeted to the c-*MYB* proto-oncogene was used to purge marrow autografts that were administered to patients with allograft-ineligible chronic myelogenous leukaemia (CML). $CD34^+$ marrow cells were purged

with ODN for either 24 ($n = 19$) or 72 ($n = 5$) hours (FIG. 4). Post-purging, c-*MYB* mRNA levels declined substantially in ~50% of patients. Analysis of *BCR–ABL* (breakpoint cluster region–Abelson murine leukaemia viral oncogene homologue) expression in a surrogate stem-cell assay indicated that purging had been accomplished at a primitive cell level in >50% of patients. Cytogenetics were evaluated at day 100 in surviving patients who did not require administration of unpurged 'rescue' marrow for engraftment ($n = 14$). (All purging protocols require storage of untreated marrow as a 'back-up', in case the purged material does not engraft.) Whereas all patients were ~100% Ph^+ (Philadelphia chromosome positive) pre-transplant, two patients had complete cytogenetic remissions, three patients had <33% Ph^+ metaphases and eight remained 100% Ph^+. The marrow of one patient yielded no metaphases, but fluorescence *in situ* hybridization (FISH) evaluation ~18 months post-transplant revealed that ~45% of cells were *BCR–ABL*$^+$, indicating that six

Table 1 | **Summary of recently published clinical trials with nucleic-acid drugs**

Target	Type of study	No. of patients	Diagnosis	Dose range	Treatment duration	Administration	Remissions	Refs
ICAM-1	Multicentre; placebo controlled; double blind	75	Crohn's disease	0.5 mg	2 days–4 weeks	SC	Not significant	106
	Placebo controlled; double blind	20	Crohn's disease	0.5–2 mg kg^{-1}	26 days	2 hours IV infusion	47% steroid-free remissions	6
PKC-α	Phase I	36	Advanced cancer	0.15–6 mg $kg^{-1}d^{-1}$	3 days per week for 3 weeks every 4 weeks	2 hours IV infusion	2 CR	82
	Phase I	21	Advanced cancer	0.5–3 mg $kg^{-1}d^{-1}$	21 days every 4 weeks	Continuous IV infusion	3 responses	85
BCL2	Phase I	21	Relapsed NHL	4.6–195.8 mg m^{-2} d^{-1}	14 days	Continuous SC infusion	1 CR, 2 minor responses	83
BCL2 combined with dacarbazine	Phase I/II	14	Advanced malignant melanoma	0.6–6.5 mg kg d^{-1}	14 days every 4 weeks	Continuous IV infusion	1 CR, 2 PR 3 minor responses	91
BCL2 combined with mitoxantrone	Phase I/II	26	Metastatic prostate cancer	0.6–5 mg kg^{-1} d^{-1}	14 days every 28 days	Continuous IV infusion	2 decreases in PSA	84
Fomivirsen CMV	Multicentre; randomized; prospective	29	CMV retinitis in AIDS patients	165 μg	Once per week	Intravitreously	Time to progression 71 versus 13 days	147
h-RAS	Phase I	23	Advanced cancer	0.5–10 mg kg^{-1} d^{-1}	14 days every 3 weeks	Continuous IV infusion	4 stable	96
c-RAF kinase	Phase I	34	Advanced cancer	1–5 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	2 stable diseases	119
	Multicentre Phase II	22	SCLC and NSCLC	2 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	No responses	148
	Phase I	22	Advanced cancer	6–30 mg kg^{-1} d^{-1}	Weekly	24 hours IV infusion	No responses	99
c-MYC	Multicentre; placebo controlled	78	After PTCA	1–24 mg d^{-1}	Single dose	intracoronary	No responses	108
	Placebo controlled	85	After coronary-stent implantation	10 mg d^{-1}	Single dose	Intracoronary	No responses	109
IGF1R	Pilot study	12	Malignant astrocytoma	2 mg 10^{-7} cells	6 hours	*Ex vivo*	2 CR 6 PR	118

AS, antisense; BCL2, B-cell lymphoma protein 2; CMV, cytomegalovirus; CR, complete remission; ICAM-1, intercellular adhesion molecule-1; IGF1R, insulin-like-growth-factor-1 receptor; IV, intravenous; c-MYC, myelocytomatosis viral oncogene homologue; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PKC-α; protein kinase C-α; PR, partial remission; PSA, prostate-specific antigen; PTCA, percutaneous transluminal coronary angioplasty; SC, subcutaneous; SCLC, small-cell lung cancer.

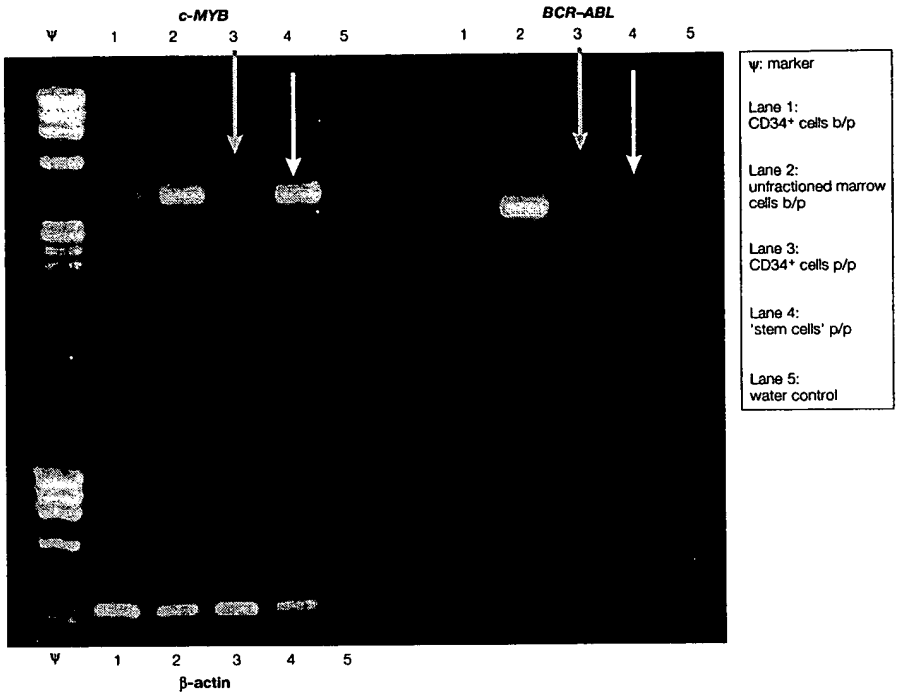

Figure 4 | **Effect of c-*MYB*-targeted ODNs on c-*MYB* mRNA expression in marrow cells.** Ethidium-bromide-stained agarose gel containing c-*MYB*, *BCR–ABL* and β-actin messenger RNA reverse transcriptase (RT)-PCR products derived from: CD34+ bone-marrow cells of a representative patient before anti-c-*MYB* oligodeoxynucleotide purging (Lane 1); unfractionated bone-marrow cells before purging (Lane 2); CD34+ cells post-purging (Lane 3); and the patient's primitive 'stem cells' post purging (Lane 4). A control RT-PCR reaction that contains only water is shown in Lane 5. Lanes containing molecular-weight markers are indicated by the symbol Ψ. Lane 3 (orange arrows) reveals that c-*MYB* mRNA is undetectable post purging, whereas some residual *BCR–ABL* expression (molecular marker of the malignant cells) persists. Efficiency of the process on primitive haematopoietic cells is shown in lane 4 (white arrows). Here, stem cells, cultured for ten days post-purge, show normal MYB expression, whereas *BCR–ABL* expression is undetectable. These data indicate that, in this patient's marrow sample, normal cells survived the purge but malignant, *BCR–ABL*-expressing cells did not. Control cells that were treated in an identical manner but not exposed to the anti-c-*MYB* oligodeoxynucleotide continue to express *BCR–ABL* (not shown), which indicates that the results are due to oligodeoxynucleotide exposure and are not a cell-culture artefact. b/p, before purging; p/p, post purging.

out of fourteen patients had originally obtained a 'major' cytogenetic response. Conclusions about clinical efficacy of ODN marrow purging could not be drawn from this small pilot study. Nevertheless, these results led the authors to speculate that enhanced delivery of ODN, targeted to crucial proteins with short half-lives, might lead to the development of more effective nucleic-acid drugs and enhanced clinical utility of these compounds in the future.

Oncogenic signal-transduction pathways

Protein kinase C-α. Protein kinase C (PKC) comprises a family of biochemically and functionally distinct phospholipid-dependent, cytoplasmic serine/threonine kinases. These proteins have a crucial role in transducing the signals that regulate cell proliferation and differentiation. PKC is overexpressed in several tumours, and antisense inhibitors of these enzymes have shown some antitumour activity *in vitro*[5,82,94] and in animal models[95]. Results of two studies that used the identical 20-mer phosphorothioate ODN against *PKCα* have been published[82,85]. The ODN was well tolerated, but antitumour effects were modest at best. Correlations with levels of *PKCα* expression were not provided.

RAS pathway

h-RAS oligonucleotide. h-RAS is a powerful regulator of several interconnected receptor-signalling pathways. The gene is constitutively active, and promotes proliferation and malignant transformation in many human tumours. Cunningham *et al.* reported results from a study that was carried out on 23 patients with various malignancies[96]. As in other studies with phosphorothioate oligonucleotides, only mild toxicities were observed. No complete or partial responses were achieved. Four patients had stabilized disease for 6–10 cycles of treatment.

c-RAF kinase. RAF proteins are crucial effectors in the RAS signal-transduction pathway. Constitutive activation of the RAS pathway is thought to contribute to malignant transformation in many cell types, which makes elements of this signalling pathway attractive targets for inhibition. Effectiveness of an antisense oligonucleotide against c-*RAF* has been shown both *in vitro*[97] and in an *in vivo* tumour-xenograft model[98]. On the basis of this work, three clinical trials were initiated[99,119,148]. A total of 78 patients were treated. No major tumour responses were documented, but some patients had stabilization of their disease.

Ribozymes

Ribozymes have been the subject of several authoritative reviews[41,100]. Although there is a comprehensive literature that describes the use of these molecules to target a wide variety of mRNA species in various cell-free, cell-intact and animal-model systems (see REFS 41,111), there is little recently published material on the use of these materials in clinical trials. The earliest clinical use of ribozymes was in patients with HIV[77,78,101,102]. As is true of antisense oligodeoxynucleotides, the approach was found to be safe when ribozymes were expressed in cells that were then delivered back to patients, but clinical efficacy was found wanting. At present, several Phase I/II clinical trials with exogenously delivered synthetic ribozymes are in early-phase clinical evaluation for patients with breast cancer, colon cancer and hepatitis. Results of these clinical investigations are anxiously awaited.

Studies in non-malignant diseases

Inflammatory diseases. Antisense oligonucleotides have been explored as anti-inflammatory agents. An example is the targeting of intracellular adhesion molecule-1 (ICAM-1) in Crohn's disease. In response to inflammatory stimuli, many cells upregulate the expression of *ICAM1*, which has an important role in the transport and activation of leukocytes. It has been shown *in vitro* and *in vivo* that administration of antisense oligonucleotides against *ICAM1* causes a decrease in receptor expression, which in turn ameliorates inflammatory reactions[103–105]. Two clinical trials with this compound in patients with Crohn's disease have been reported[6,106]. In the double-blind study reported by Yacyshyn *et al.*[6], 20 patients were randomized to receive a saline placebo or anti-*ICAM1* antisense oligonucleotide. The treatment was well tolerated, and after 6 months, disease remission was reported in 47% of patients in the antisense group compared with 20% of patients in the placebo group. Furthermore, corticosteroid usage was significantly lower ($p = 0.0001$) in the antisense-treated patients. These results engendered a great deal of excitement, but the enthusiasm was subsequently dampened by the follow-on study that was carried out with this compound in a larger group of patients with this disease ($n = 75$)[106]. In this placebo-controlled study, no statistically significant differences in steroid use between the treatment or placebo groups was observed, although 'positive trends' were seen in the patients who were treated with the antisense oligonucleotide. As with other studies, toxicity was mild and consisted primarily of pain at the injection site, fever and headache.

The anti-*ICAM1* oligonucleotide has also been evaluated in patients with psoriasis. The drug was initially administered by intravenous infusion to these individuals, but examination of their skin indicated that delivery to its various layers was poor. For this reason, a topical formulation was developed. Although preclinical data about uptake of this formulation into the skin and downregulation of expression of the target were encouraging[107], the ensuing clinical trial showed only modest, short-term effects in these patients (see the ISIS Pharmaceuticals web site online). The ultimate usefulness of this compound remains to be determined.

Cardiovascular disease. RESTENOSIS of coronary vessels after *trans*-catheter re-vascularization procedures remains a serious clinical problem. Manipulation of coronary vessels invariably leads to endothelial-cell injury, which is often accompanied by thrombosis, smooth-muscle-cell activation and subsequent vascular remodelling. The myelocytomatosis viral oncogene homologue (c-MYC) has been identified as an important mediator in this process through its effects on regulating the growth of vascular cells in atherosclerotic lesions. Accordingly, it has been postulated that c-*MYC* might make an attractive target for preventing post-angioplasty complications, and at least two clinical trials using a 15-mer phosphorothioate-modified antisense ODN against c-*MYC* have been reported[108,109]. Both studies showed safety of intracoronary application of the drug, but no objective clinical responses.

Oligonucleotides as immunological adjuvants

Over the past several years, it has become increasingly appreciated that several types of immune cell have pattern-recognition receptors that can distinguish prokaryotic DNA from vertebrate DNA[110]. This is apparently accomplished by the ability of these receptors to recognize unmethylated CpG dinucleotides in certain base contexts (CpG motifs)[111]. Bacterial DNA, or more germane to this discussion, synthetic oligodeoxynucleotides that contain these unmethylated CpG motifs, can activate immune responses that have evolved to protect the host against infections. Responses of this type are similar to T-helper type 1 (T_H1)-cell responses, and lead to activation of natural killer (NK) cells, dendritic cells, macrophages and B cells[112]. CpG DNA-induced immune activation has been shown to protect certain hosts against infection, either alone, or in combination with vaccines. It is reasonable to suppose, then, that CpG-containing oligonucleotides might prove to be effective adjuvants for the immunotherapy of cancer, and for boosting immune responses to antigens that are less efficient in this regard, but to which one would like to immunize a host[113].

The most recent application of this principle was reported in abstract form at the December 2001 meeting of the American Society of Hematology, where preliminary results from a clinical trial in which the

RESTENOSIS
A reduction in lumenal size after an inter-arterial coronary intervention.

Table 2 | **Current and planned clinical trials with antisense oligonucleotides and ribozymes**

Product	Diseases	Company
Anti-c-MYC (AS)	Cardiovascular restenosis, Phase II	AVI Biopharma
EPI 2010 (AS against adenosine A1 receptor)	Asthma, Phase II	EpiGenesis Pharmaceuticals
Genasense (AS against BCL2)	Haematological malignancies Solid tumours, Phase III	Genta
GTI 2040 (AS against ribonucleotide reductase)	Solid tumours, Phase I and II	Lorus Therapeutics
HGTV (AS against HIV)	HIV, Phase II	Enzo Biochem
CpG molecules	Solid tumors Infectious diseases, Phase I/II	Coley Pharmaceutical Group
Angiozyme (Ribozyme against VEGFR1)	Breast and colon cancer, Phase II	Ribozyme Pharmaceuticals
Heptazyme (Ribozyme against HCV)	HCV, Phase I	
Herzyme (Ribozyme against HER2)	Breast and ovarian cancer, Phase I	
ISIS 3521 (PKC-α) ISIS 5132 (c-RAF) ISIS 2503 (h-RAS) G 3139 (BCL2) GEM 231 (PKA)	NSCLC, NHL, Phase III Solid tumours, Phase II NSCLC, Phase II NHL, Phase II/III PKA, Phase II	ISIS Pharmaceuticals

AS, antisense; BCL2, B-cell lymphoma protein 2; CpG, unmethylated CpG dinucleotides; HCV, hepatitis C virus; HER2, tyrosine-kinase growth-factor receptor, also called c-ERBB2; HIV, human immunodeficiency virus; c-MYC, myelocytomatosis viral oncogene homologue; NHL, Non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PKA, protein kinase A; PKC-α; protein kinase C-α; VEGFR1, vascular-endothelial-growth-factor receptor 1.

safety and efficacy of a CpG adjuvant was investigated in 16 patients with non-Hodgkin's lymphoma were reported[114]. Analysis of the data accrued at the time of submission indicated that the oligonucleotide increased the number and activity of NK cells in treated patients, and 2 out of 16 treated patients achieved partial remission. The study is continuing, and a follow-on trial of the CpG oligonucleotide in combination with rituximab is being planned.

Problems in need of solution

Nucleic-acid-mediated gene silencing has been used with great success in the laboratory[107,115–117], and this strategy has also generated some encouraging results in the clinic[90,93,96,118,119]. Nevertheless, it is widely appreciated that the ability of nucleic-acid molecules to modify gene expression *in vivo* is quite variable, and therefore wanting in terms of reliability[120,121]. Several issues have been implicated as a root cause of this problem, including molecule delivery to targeted cells and specific compartments within cells, and identification of sequence that is accessible to hybridization in the genomic DNA or RNA[2]. Intuitively, DNA accessibility is limited by compaction of nuclear material and transcription activity of the gene target. Formal approaches for solving this problem have not been widely discussed. In mRNA, sequence accessibility is dictated by internal base pairing and the proteins that associate with the RNA in a living cell. Attempts to accurately predict the *in vivo* structure of RNA have been fraught with difficulty[122]. Accordingly, mRNA targeting is largely a random process, which accounts for the many experiments in which the addition of an antisense nucleic acid yields no effect on expression. Several approaches to this problem have been tried, including trial-and-error 'walks' down the mRNA[123], computer-assisted modelling of RNA structure[124], hybridization of RNA to random oligonucleotides arrayed on glass slides[125,126] and variations on the theme of using random oligonucleotide libraries to identify RNase H cleavable sites, in the absence or presence of crude cellular extracts[127,128]. Recent work from this laboratory indicates that self-quenching reporter molecules might be useful for solving *in vivo* RNA structure[129], but the reliability and usefulness of this approach remain to be proven.

Another problem in this field is the limited ability to deliver nucleic acids into cells and have them reach their target[120]. Without this ability, it is clear that even an appropriately targeted sequence is not likely to be efficient. As a general rule, oligonucleotides are taken up primarily through a combination of adsorptive and fluid-phase endocytosis[130,131]. After internalization, confocal and electron microscopy studies have indicated that the bulk of the oligonucleotides enter the endosome–lysosome compartment, in which most of the material becomes either trapped or degraded. Biological inactivity is the predictable consequence of these events. Nevertheless, oligonucleotides can escape from the vesicles intact, enter the cytoplasm and then diffuse into the nucleus, where they presumably acquire their mRNA, or in the case of decoys, protein target[131,132–134]. Delivery technologies continue to improve, so it is likely that present methods, and/or other evolving technologies, will be used successfully to deliver optimized nucleic acids to their cellular targets[135,136]. Indeed, it is our hypothesis that development of

effectively targeted and efficiently delivered nucleic-acid molecules will lead to important advances in the diagnosis and treatment of human malignancies[93], and other diseases for which this class of molecule has been proposed to be effective.

In addition to delivering and targeting oligonucleotides to the mRNA, we believe that other considerations might improve the efficacy of this strategy. In this regard, we suggest that the abundance and half-life of the target mRNA should also be considered when selecting a gene target. The c-*MYB* mRNA that we have chosen to target, as well as its encoded protein, has an estimated half-life of ~30–50 minutes[137,138]. By contrast, BCL2, for example, has a half-life that has been estimated at ~14 hours[139], and RAF and RAS have half-lives that are estimated to be >24 hours[140,141]. Attempts to eliminate these proteins from cells using oligonucleotides might therefore prove more difficult. Whether these considerations will apply to extremely long lived or endogenously expressed antisense vectors, remains to be seen. As the efficiency of these molecules for perturbing gene expression improves, an important consideration in target selection will be the relative selection in the target versus non-targeted tissue. The ability to target genetic polymorphisms, or cells affected by loss of heterozygosity, might be an effective solution to this problem[142]. Finally, another approach for improving the effectiveness of nucleic-acid drugs as anticancer agents that is under intense investigation is to combine them with more traditional therapeutic modalities. Although this might well prove useful, we strongly believe that it remains important to continue to explore strategies that are designed to promote more reliable and efficient gene silencing with oligonucleotides alone. As discussed above, a prime motivating force for developing these drugs is the hope for non-toxic therapies. Adding back chemotherapy, although perhaps useful in the short term, is in the end counter-productive to this specific goal, unless it can be used at significantly reduced dosages. So far, this has not been the case.

Conclusions

The concept of inhibiting gene expression with antisense nucleic acids developed from studies that were initiated almost a quarter of a century ago[13,14]. Despite the fact that the mechanism by which these molecules modulate gene expression is not always certain[12,128,143], clinical development of antisense compounds has proceeded to the point at which several nucleic-acid drugs have entered Phase I/II, and in a few cases, Phase III trials. Others are about to begin, or are in the late planning stages (TABLE 2). The original motivation for developing these molecules remains strong. The recent development of leukaemia cells that are resistant to the small-molecule inhibitor Gleevec provides another incentive. Although a cell might be able to evolve mutated proteins that evade a small-molecule protein inhibitor, this cannot happen if the mRNA that encodes that protein is no longer made. Accordingly, although only one antisense drug has received FDA approval so far[144], all of the investigators who have laboured long and hard in this field hope that the time to celebrate significant achievements in the clinic will shortly be forthcoming.

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Online links

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The pain of antisense: in vivo application of antisense oligonucleotides for functional genomics in pain and analgesia

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Abstract

As the genomic revolution continues to evolve, there is an increasing demand for efficient and reliable tools for functional characterization of individual gene products. Antisense oligonucleotide-mediated knockdown has been used successfully as a functional genomics tool in animal models of pain and analgesia yet skepticism regarding the validity and utility of antisense technology remains. Contributing to this uncertainty are the lack of systematic studies exploring antisense oligonucleotide use in vivo and the many technical and methodological challenges intrinsic to the method. This article reviews the contributions of antisense oligonucleotide-based studies to the field of pain and analgesia and the general principles of antisense technology. A special emphasis is placed on technical issues surrounding the successful application of antisense oligonucleotides in vivo, including sequence selection, antisense oligonucleotide chemistry, DNA controls, route of administration, uptake, dose-dependence, time–course and adequate evaluation of knockdown.

Keywords: Knockdown; Central nervous system; Rat; Mouse; Gene targeting; Intrathecal; Animal model; Nociception

Contents

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1. Introduction

Antisense oligonucleotide (ASO)-mediated knockdown of gene expression has contributed substantially toward our understanding of the physiology and pathobiology of pain and analgesia. ASO use has many advantages including the ability to specifically target a single member of a large and/or highly homologous family and the ability to control the timing of protein knockdown. ASO technology therefore enables us to elucidate the role of a target protein in either the induction or maintenance of pathological processes by beginning the ASO treatment before or after induction, respectively.

ASOs are particularly well suited for the validation of new drug targets because the functional effects of ASO-mediated knockdown of a prospective drug target can predict its therapeutic potential. Through genomics we have increased our understanding of many diseases at the molecular level, yet the functional and therapeutic implications of much of the information remain unexplored. The need for efficient and reliable tools for the validation of potential new drug targets has become a critical issue in the drug discovery process.

ASO-based approaches have been successfully applied to many classes of pain-relevant genes including G-protein coupled receptors (GPCRs), voltage- and ligand-gated ion channels, neuropeptides and second messengers. However, despite the many important contributions, skepticism about the validity and utility of ASOs as a functional genomics tool remains. The general impression that the antisense technology, although initially very promising, has not lived up to expectations is somewhat justified. In looking at the number of Medline citations (Fig. 1) since 1985 in response to a keyword search

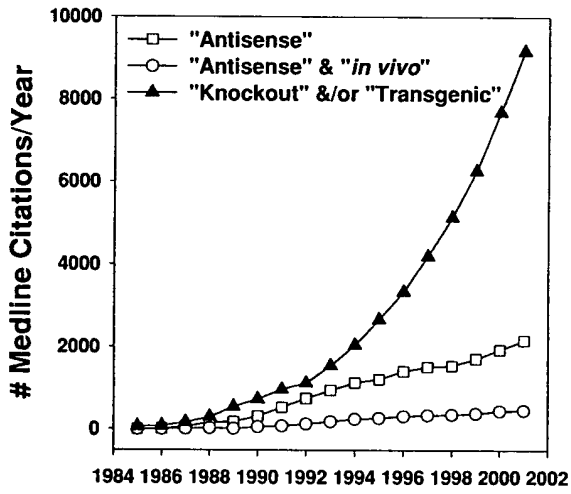

Fig. 1. Number of Medline citations containing the keywords "antisense", "antisense and in vivo", or "knockout or transgenic". The number of antisense related citations has remained relatively constant for more than a decade. In the same time period, the number of publications containing the terms "knockout" and/or "transgenic" have risen rapidly.

for "knockout" or "transgenic" we see a doubling every 2–3 years, suggesting continued increased utilization of the method. In contrast, examination of the number of Medline citations for "antisense" reveals minimal growth by comparison. Limiting the number of "antisense" citations to those with reference to "in vivo" produces a nearly flat line. This under-representation in the literature could be interpreted as indicative of fundamental flaws in the antisense technology. On the contrary, we suggest that the apparent under-utilization of ASOs is symptomatic of the many technical and methodological challenges of ASOs use and the relative lack of systematic studies delineating the best practices for in vivo applications. The goal of this review is therefore to outline the contribution of antisense studies to the field of pain and analgesia, the general principles of antisense technology, and the many technical issues surrounding the application of ASOs in vivo.

2. Antisense contributions to in vivo pain studies

The rationale driving the application of ASOs in pain studies generally falls into several major categories. First, ASOs enable the independent functional characterization of a single member of a closely related family of proteins that cannot be specifically targeted using pharmacological tools. Second, ASOs can be used to examine the molecular basis of pharmacologically defined subtypes (for example, see the discussion on opioid receptors in Section 2.2). Third, ASOs provide a tool for determining the mechanism of action of pharmacological agents. Fourth, as a functional genomics tool for target identification and validation, the antisense technology offers a screening method for potential new therapies and its use can streamline and accelerate the drug discovery process. Finally, ASOs have the potential to serve as therapeutic agents.

The studies highlighted in this section illustrate the wide variety of pain-relevant proteins that have been targeted with ASOs. Many of these studies have made significant contributions to our understanding of the neural mechanisms of pain and analgesia. This overview is not intended as a comprehensive tally of all the pain-relevant ASO-based reports published to date, nor does it include studies of pain-relevant genes where the experimental endpoints did not include nociception. The specific details of the experimental design, the evaluation of knockdown and the pain models used in selected studies are itemized in Table 2 and Section 5.

2.1. Ion channels

2.1.1. P2X receptors

The P2X purinergic receptors (ATP-gated cation channels) can signal tissue damage when activated by ATP released from dying cells [1,2]. The P2X3 subunit in particular has been implicated in acute and chronic pain since its discovery [3–5], largely due to its localization in nociceptive sensory neurons [6–9]. However, the lack of specific pharmacological tools prevented the generation of direct evidence to support this contention. Studies using knockout mice reported a lack of involvement of P2X3 in normal somatic pain thresholds but hinted at some involvement in the signaling of more tonic stimuli [10,11]. It was not until the publication of two studies successfully targeting P2X3 with ASOs that the importance of this subunit in chronic neuropathic and inflammatory pain was demonstrated directly [12,13]. In inflammatory models, P2X3 appeared to be involved in Complete Freunds Adjuvant (CFA) but not carrageenan-induced thermal hyperalgesia, and the ability of P2X3 ASO-treatment to attenuate CFA-induced hyperalgesia correlated with the magnitude of the knockdown obtained in each animal [13]. Knockdown of P2X3 attenuated mechanical allodynia (assessed by von Frey filaments) induced by spinal nerve ligation [13] but not mechanical allodynia induced by partial sciatic nerve injury [12]. However, the development and maintenance of mechanical hyperalgesia following partial sciatic nerve injury, measured using an analgesymeter (Ugo-Basil, Milan, Italy), was attenuated by P2X3 knockdown [12]. In contrast, P2X3 knockout mice show impaired thermal but not mechanical allodynia following nerve injury, suggesting that the P2X3 receptor is not involved in mechanical pain [14]. These inconsistencies could be due to strain, species or methodological differences. Despite these discrepancies, however, ASO approaches have clearly provided direct

evidence for a role of P2X3 receptors in chronic pain.

2.1.2. Voltage-gated sodium channels

Nerve injury results in an increased density of voltage-gated Na^+ channels in the damaged nerve, leading to abnormal excitability [15]. The relative importance of the members of the voltage-gated Na^+ channel family, NaV1, in chronic pain remains a subject for debate. Although there are no pharmacological tools available for the selective targeting of each subtype, based on their sensitivity to tetrodotoxin (TTX) the channels have been classified into two functional categories: TTX-sensitive (TTX-s) or TTX-resistant (TTX-r). The enhanced Na^+ currents in injured axons are largely mediated by TTX-r channels [16]. The NaV1.8 channels, in being both TTX-r and upregulated in injured nerves [17], may be important contributors to this phenomenon. Indeed, ASOs targeting NaV1.8 eliminate the TTX-r component of the C-wave in injured sciatic nerves [18]. Behaviorally, ASOs directed at NaV1.8 have been shown to inhibit both the development and maintenance of nerve injury-induced thermal hyperalgesia and mechanical allodynia [19,20]. In a visceral pain model, NaV1.8 knockdown reduced the hyperexcitability produced by acetic acid treatment in the bladder [21]. Interestingly, as was observed with P2X3, knockdown of NaV1.8 attenuated the behavioral signs of CFA- but not carrageenan-induced inflammatory pain. There may be a fundamental difference in these two models that makes the latter less susceptible to ion channel downregulation. It has been speculated that CFA produces both inflammatory and nerve injury related effects, which, if true, would be consistent with sensitivity of the nerve injury component to the downregulation of P2X3 and NaV1.8.

2.1.3. Other ion channels

Antisense studies targeting other families of ion channels have resulted in the following observations: (1) the NMDA receptor subtypes R1 and R2C are important in the etiology of formalin-induced nocifensive behaviors whereas the R2D is not [22,23]; (2) the $5HT_3$ receptor is important in 5HT-mediated spinal analgesia [24]; (3) the α4 subunit of the nicotinic acetylcholine receptor, nAchR, plays a critical role in nicotinic agonist-induced analgesia [25] and (4) Kv1.1 potassium channels are likely to be involved in morphine, baclofen and clonidine antinociception but not in the antinociception induced by H1-antihistamines [26–28]. This pattern of involvement of Kv1.1 is consistent with the mechanism of action of these compounds (i.e., morphine, baclofen and clonidine are agonists at inhibitory receptors whereas antihistamines are antagonists at an excitatory receptor).

2.2. G-Protein coupled receptors

2.2.1. Opioid receptors

An enormous amount of progress has been made in the opioid receptor (OR) field because of antisense technology. Although there are now knockout mice available, ASO-based methodologies pre-date their development and have been applied to a wide range of questions including: identification of functional subtypes, splice variants, and ligand selectivity. There has been an inconsistency in the literature with regard to the OR subtypes in that the pharmacologically-identified subtypes of mu-, delta- and kappa-opioid receptors (δOR, μOR, and κOR) outnumber the cloned receptors MOR1, DOR1 and KOR1. Targeting of the cloned ORs with antisense has supported the existence of pharmacological subtypes at the molecular level. For example, the antinociception elicited by the δOR agonists DPDPE and deltorphin II displays differential sensitivity to ASOs targeting the cloned DOR1 receptor, consistent with previous evidence for the two pharmacological subtypes, δOR-1 and -2 [29–33]. Similarly, the actions of a κOR agonist in the modulation of cutaneous versus visceral nociception were differentially sensitive to KOR antisense treatment, suggesting the presence of an additional, non-KOR1 site of action for this agonist in viscera [37]. ASO-based studies have also supported a role for DOR1 in morphine tolerance, dependence and stress-induced analgesia [34–36]. Finally, ASOs have been used to "map" splice variants by the differential targeting of exons [30]. In this manner it was determined, for example, that different splice variants of the cloned mu-opioid receptor MOR1 display differential sensitivity to a plethora of μOR agonists [38–46].

2.2.2. α_2-Adrenergic receptors

Antisense studies investigating the relative contributions of the α_2-adrenergic receptor (α_2AR) subtypes α_{2A}AR and α_{2C}AR in α_2-adrenergic agonist-induced analgesia are consistent with the knockout studies demonstrating that most [47–49], but not all [50] of the α_2-adrenergic agonists require the α_{2A}AR for analgesic efficacy. In fact, ASO treatment and knockout mice were used in the same study to provide convergent evidence for the involvement of the α_{2C}AR in the analgesic action of the α_2AR agonist moxonidine [50].

2.2.3. Metabotropic glutamate receptors

The therapeutic potential of metabotropic glutamate receptor antagonists and lack of subtype-selective agents have prompted the evaluation of the role of the mGluR1 subtype in pain and analgesia models using ASOs. In acute pain assays, mGluR1 ASO treatment produced analgesia and decreased neuronal activity in response to noxious stimuli but did not alter responses to innocuous stimuli [51]. In chronic pain models, reduction of mGluR1 attenuated nerve injury- and CFA-induced thermal hyperalgesia and mechanical allodynia [52,53]. In addition, some restoration of morphine efficacy was observed in neuropathic rats [52] and mGluR1 ASO treatment attenuated the development of morphine tolerance [54]. Based on these data, mGluR1 antagonists would be expected to reverse hyperalgesia and allodynia and increase nociceptive thresholds and could therefore be effective therapeutic agents. In addition, co-administration of mGluR1 antagonists with morphine may enhance analgesic efficacy and reduce the development of morphine tolerance.

2.2.4. Other G-protein coupled receptors

Several galanin receptor subtypes have been cloned, raising questions regarding their role in different aspects of galaninergic transmission. Antisense studies targeting GalR1 have demonstrated that this subtype is critical for galanin-mediated inhibition of C-fiber-induced facilitation of nociceptive reflexes [55,56]. Thus, GalR1 may mediate the analgesic actions of galanin receptor agonist in the spinal cord. GPCR antisense studies have also shown that the $5HT_{1B}$ serotonergic receptor subtype may not be required for 5HT-mediated analgesia [24] and that cholecystokinin (CCK) has anti-opioid activity at the CCK_B receptor [57]. Other GPCRs which have been successfully targeted with antisense in pain-relevant studies include: muscarinic M1- [58,59], dopamine D2- [60], neurotensin NT1- [61–63] and the cannabinoid CB1-receptors [64,65].

2.3. G-Proteins

Stimulation of G-protein coupled receptors can lead to the activation of multiple G-protein α-subunits (G_α) [66]. Given the functional consequences of differential G_α activation, it is important to understand which of these subtypes is relevant to signaling in vivo. Many of the G_α subunits have been targeted with ASOs and these studies have resulted in some interesting insights into G-protein coupling. Most relevant to pain and analgesia are the studies investigating the complement of G_α subunits activated by specific analgesics. Activity of the supraspinally administered μOR agonists morphine, DAMGO and sufentanil, for example, was attenuated by antisense targeting $G_{i2\alpha}$, but not $G_{i1\alpha}$, $G_{i3\alpha}$ or $G_{s\alpha}$ [67]. In addition, $G_{i2\alpha}$ activation differentiated between morphine analgesia and other effects of morphine such as acute dependence and constipation [68]. It has also been shown that different μOR and δOR agonists are differentially sensitive to knockdown of a given G_α subunit. For example, morphine and morphine-6 β-glucuronide appeared to utilize different G_α subunits [69] as did agonists acting at the δOR-1 and δOR-2 subtypes [70]. Furthermore, analgesics acting at other families of inhibitory GPCRs (i.e., clonidine at α_2ARs) shared G-proteins with some ORs and not with others. For example, Karim and Roerig demonstrated that spinal analgesia elicited by clonidine and morphine was attenuated by knockdown of different G_α subunits [71]. Many of these studies have been reviewed elsewhere [72,73]. In addition to selective knockdown of G_α subunits, the G protein subunit γ2 has been targeted with ASOs, revealing a significant role for this protein in antinociception induced by i.c.v. administration of the δOR agonist DPDPE [74].

2.4. Miscellaneous signaling molecules, growth and transcription factors

In addition to the functional roles of many ion channels, GPCRs and G-proteins, ASOs have been applied to a wide range of pain-relevant questions such as morphine tolerance. It has been shown, for example that knockdown of β-arrestin attenuates both morphine tolerance and nerve-injury induced allodynia [75], that knockdown of the nNOS splice variants attenuates the development of morphine tolerance (nNOS-1) or reduces morphine analgesia (nNOS-2) [76] and that PKCα is involved in the development of morphine tolerance. Other molecular classes successfully targeted with antisense include growth and transcription factors. For example, ASO targeting helped identify the role of neurotrophin-3 in nerve-injury induced sprouting [77] and showed that knockdown of c-fos can inhibit inflammation-induced regulation of neuropeptide gene expression [78].

3. Mechanism of action

Antisense inhibition of gene expression relies upon the rules of nucleic acid base pairing. An ASO, typically 15 to 25 nucleotides in length, is designed to bind to a complementary sequence on the target RNA. As a consequence, the protein product coded by that particular RNA is not synthesized. The mechanisms by which ASOs inhibit protein expression fall into two major categories: cleavage of mRNA by activation of RNase H and steric blockade [79,80]. The contribution of these mechanisms to the action of individual ASOs is influenced by their chemistry and the location of the targeted sequence within the RNA molecule.

RNase H is an enzyme that degrades the RNA strand of a DNA–RNA duplex. Upon binding of ASOs to RNA, an RNA–DNA duplex is formed and RNase H digests the RNA that the antisense compound has hybridized with. As the original antisense compound remains intact, the ASO is free to target and bind with another strand of RNA. This process can be repeated over and over, allowing one oligonucleotide (ON) to cause the cleavage of multiple RNAs [79,80]. Two types of mammalian RNase H enzymes have been identified: RNase H1 is thought to participate in replication whereas RNase H2 may play a role in transcription [81]. The limited information available on the relative contribution of the two enzymes to antisense effects suggests that RNase H2 is the major player and its cellular localization is likely to differ in different cell types [82]. The relevance of RNase H-dependent degradation of ASO-targeted genes in the central nervous system (CNS) has been addressed in a study showing that the in vivo activity of ASOs against CRF2 was lost when the ASOs were modified to no longer support RNase H activation [83]. However, the expression, activity and role of RNase H in neurons have not been examined directly.

Non-RNase H-dependent mechanisms of ASO-mediated knockdown are typically ascribed to steric blockade of RNA. Binding of an ASO to RNA has been proposed to interfere with numerous RNA processing events, possibly leading to one or more of the following: inhibition of 5′-capping, modulation of splicing, inhibition of 3′-polyadenylation, translational arrest and the disruption of critical RNA structure(s) [79,80,84,85].

On a practical level, it is important to be aware of the different mechanisms of ASO action. First, some DNA analogues result in the formation of ASO–RNA duplexes which are not substrates for RNase H. Thus, a sequence may show activity when synthesized using one type of DNA analogue but not another. Second, if an ASO elicits its effects solely through steric mechanisms it may reduce expression of a target protein without affecting mRNA levels. Therefore, validation of knockdown limited to mRNA measurement would fail to detect any ASO activity. Finally, ASOs acting through non-RNase H-dependent mechanisms may have effects other than knockdown of expression. Examples include inhibition of the expression of one splice variant while enhancing the expression of another [85] and potentiation of expression of a targeted gene through increased RNA stability [84].

4. Comparison to other gene and protein targeting methods

Alternative technologies for inhibition of protein expression or function include the use of ribozymes, RNAi, viral vectors, sequestering antibodies and

knockout and/or transgenic animals. Ribozymes are somewhat analogous to ASO in that they rely on complementary base pairing for targeting and have the ability to control both the onset and termination of the treatment period. However, unlike ASOs, they also act as enzymes, directly cleaving the target RNA. The use of ribozymes in vivo is largely unexplored. The potential of RNAi for long-term gene silencing has been demonstrated in mammalian systems in vitro [86,87] and in vivo [88] has stirred excitement about its application as a functional genomics tool. However, we are very much at the beginning of exploring the utility of RNAi, particularly in the CNS. Direct comparison of RNAi- and ASO-mediated knockdown suggested many similarities between the two approaches [89]. Use of viral vectors to modulate gene function continues to grow in popularity as the methodologies become more approachable. Viral vectors have been applied successfully in pain-related studies (for a review see Ref. [90]). One disadvantage of this approach is the inability to terminate activity, although this limitation may become less relevant with continued advances in gene delivery methodologies. Sequestering antibodies have been used successfully in pain models (for example see [91]) but their availability is limited.

Currently the most common method for the evaluation of gene function in vivo is the use of knockout and/or transgenic animals. Table 1 outlines some of the characteristics of antisense technology in comparison to knockout and/or transgenic models. This comparison is not meant to favor one method over the other but rather to present some of the relative advantages and disadvantages of each. The ongoing debate regarding the superiority of knockdown vs. knockout and other methodologies should be tempered by the following realizations:

- All methods have inherent difficulties and limitations.
- The decision to pursue one particular experimental approach over others depends on many complex, case-specific factors.
- Alternative methodologies should be viewed as complementary.

5. Experimental design of in vivo antisense-based studies

As is true with any method, success requires a good understanding of the technical advantages and limitations of the technology and problems are nevertheless inevitable. Many groups have invested resources in ASO-based experiments and have been frustrated by uninformative, uninterpretable, misleading or simply irreproducible results. The failure of the ASO technology to live up to its potential can be attributed in large part to the plethora of technical

Table 1
Knockdown versus knockout/transgenic

Antisense technology	Knockout/transgenic technology
Applicable in a wide range of species, including humans	Currently limited to a few species (i.e., mice, pigs)
Effects are fully reversible	Effects are not generally reversible with current technology
Less expensive	More expensive
Rapid: experiments can begin within weeks of conception	Development can be very slow, particularly when accounting for back-crosses
Does not require use of special equipment or facilities	Requires use of a lot of specialized facilities
No confounding developmental effects	Potential exists for confounding development effects
Some risk for confounding compensatory effects	High risk for confounding compensatory effects
Potential for simultaneous targeting of multiple genes	Targeting of multiple genes possible but laborious
Usually only achieves partial knockdown	Total obliteration of gene product
Animals must be generated de novo for each experiment but once a protocol is established it can be easily adapted to other targets	Once a line is established the number of subjects is restricted only by breeding and genotyping expenses but is limited to one target
Need to control for non-specific DNA effects	Need to control for developmental and compensatory effects
High doses of antisense can have toxic effects	Knockout of some genes produces a lethal phenotype
Cannot generate over-expressers	Generation of over-expressers is common
Can be used to target a gene before, during or after an experimental manipulation	Although under development, conditional knockout technology is not yet mainstream

issues that must be addressed when designing an ASO-based experiment. Among the critical issues are sequence activity and selectivity, ON stability, proper use of controls, route of administration and uptake, dose-dependence, time–course and adequate evaluation of knockdown. Careful consideration of all these technical aspects is essential for successful ASO-based studies. Table 2 presents examples of experimental design parameters, the resultant knockdown in expression and the in vivo models used for selected pain-relevant studies from the discussion in Section 2. It is intended to illustrate the many variations in target class, design, application and efficacy of knockdown. The abbreviations used in Table 2 are defined in the relevant topical segments throughout Section 5.

5.1. Antisense oligonucleotide sequence selection

Of the many factors influencing the success of antisense treatment, identification of active ASO sequence(s) is the most critical and cannot be overemphasized. Improved methodologies can only potentiate the activity of weaker sequences that would otherwise fall below detection levels [92]. Estimates suggest that between 5 and 15% of randomly selected ASOs will be sufficiently active at a target gene to generate meaningful results [93–96]. This is largely due to the inaccessibility of most of the RNA molecule for ASO binding as a consequence of its complex secondary structure. Furthermore, associations with cellular proteins will render additional regions unavailable for binding. Although there are methods that increase the probability of finding active sequences, the current state of knowledge is such that at some level sequence identification remains a hit or miss process.

A comprehensive review of the literature by Tu et al., showed that 82% of published antisense studies (up to September 1997) reported data from just a single ASO [96]. In addition, often there is no information on the process or rationale for the selection of the sequence, suggesting that the ASO design was based on a "lucky strike" rather than a systematic approach. The seeming incongruity between this statistic and the probability of randomly hitting upon an active sequence has raised concerns about the integrity of many of those studies [93], yet this judgment may be overly harsh for several reasons: First, there is an inevitable publication bias as studies using inactive ASOs are unlikely to be published because no insights into biological processes will have been obtained. (This situation should not be confused with biologically active ASO studies in which knockdown is achieved but no phenotype is observed). Second, it is generally not common practice to report the inactive ASOs that were rejected before an active sequence was found, just as a medicinal chemist may not report all the derivatives of a compound that lack activity.

The most conservative strategy for ASO sequence identification is the process of gene walking. An in vitro assay is first established to quantify mRNA and/or protein expression of the target. A series of ASOs complementary to the target RNA are then designed by walking along the gene. The entire sequence is covered and all the ASOs are screened for biological activity. Those that show activity may then be further evaluated for specificity, potency and maximal efficacy. It is not uncommon in industrial settings for hundreds of ONs to be screened per gene. In a recent report by Sewell et al., 264 sequences were screened to identify an optimal anti-TNFα ASO [97]. One of the major hurdles encountered in this strategy is the development of an efficient screening system. This requires a suitable cell line that either endogenously expresses or can be transfected with the target gene, the optimization of ON transfection conditions in that cell line and an assay for the detection of knockdown. In addition, the expense of synthesizing the many ON sequences required by this method may be cost prohibitive.

A novel strategy developed by Hoen et al. may facilitate the in vitro evaluation of antisense sequences [95]. The authors created a green fluorescent protein (GFP) fusion construct of their target gene. This construct was then transfected into a cell line. Exposure of the cells to ASOs simultaneously reduced the expression levels of the target gene and GFP. Thus GFP serves as a reporter for ASO-mediated decreases in the expression of the target gene. Major advantages of this method are that it can be applied to any gene independent of its function and that the evaluation of knockdown (i.e., flow cytometry for GFP) is rapid, quantitative and does not require development of a novel gene-dependent

assay. However, the possibility remains that the addition of GFP may change the secondary structure of RNA, resulting in different accessible sites than the native gene.

It is important to note that in vitro efficacy does not necessarily predict nor guarantee in vivo efficacy. The RNA structure, the complement of accessory proteins and the regulation of the target gene may be different in vivo than in the in vitro screening system. This is especially true if the selection is performed using a cell free system where none of the normal RNA-binding proteins are present. Furthermore, the percent activity demonstrated in vitro might not predict the percent knockdown in vivo. Specific details such as the cell line, the type of ONs (i.e., unmodified vs. chemically modified DNA), the efficiency of uptake and the dose will all impact the level of activity in vitro. Similarly, the interaction of complex factors such as chemical modifications, dose, route of administration and diffusion will profoundly influence the degree of antisense activity in vivo. If, for example, a series of potential ASOs are tested and the best one is only able to reduce expression by a third, it is possible that the same sequence might be significantly more efficacious in vivo or under different in vitro conditions. Thus, the use of arbitrary cutoff (i.e., 50 or 75%) to discard ASOs with low in vitro activity levels may lead to the exclusion of active ASOs. The potential for discrepancies between in vivo and in vitro efficacies does not discount the possible benefits of selecting active ASOs in an in vitro system provided such a system is easily available, is optimized and models closely the cell types to be targeted in vivo (for example, using neuroblastoma cell lines for CNS targets). Under these conditions failure to detect in vivo activity of ASOs that were active in vitro would be likely due to other factors such as dose or uptake and efforts can therefore be focused on addressing these other variables.

Central to the identification of active ASOs is the characterization of accessible sites on the RNA of the target gene [92]. Computer-based strategies have been developed that involve assessment of the free energy requirements for hybridization and evaluation of computer-predicted local RNA secondary structure [98–100]. In addition, a number of empirical methods have been described, typically based on the use of random ON libraries or arrays to identify accessible regions on the target RNA [101–105]. There are also approaches for identification of active ASO that are independent of characterization of accessible sites. Several groups have performed comprehensive literature surveys to identify sequence motifs which are positively or negatively correlated with antisense activity [96,106]. In one study the sequence motif GGGA was found in a disproportionate number of successful sequences. When this motif was then used to select ASOs to a specific gene, the proportion of active ASOs increased from 2/18 when randomly selected to 18/25 [96]. Some of these computer-based tools have been made available on-line (for example, see Refs. [107,108]).

Over the years the experimental use of ASOs has resulted in the generation of "rules" for sequence design including: avoid sequences with G-quartets, avoid sequences that contain the two-base sequence CpG (cytosine–phosphate–guanine), and favor ASOs which will hybridize in proximity to the start codon. G-Quartets (i.e., four G-bases in a row) result in the formation of a quadruplex which interacts with a number of proteins, causing non-specific effects [79]. Systemically administered phophorothioate ASOs containing the sequence CpG activate mammalian B cells and natural killer cells [109]. Immune system stimulation has therefore limited their use as antisense drugs. Interestingly, this immune response is only triggered when the CpG sequence is unmethylated. It is hypothesized that because the unmethylated version is common in bacterial DNA and not in mammalian DNA, the immune system interprets the unmethylated ON sequences as bacterial and activates a response to combat the "bacterial" invasion. In an interesting twist, these properties may be exploited for possible therapeutic benefits [110,111]. It should be noted, however, that the relevance of these phenomena to centrally administered ONs is not clear. Finally, the belief that ASOs directed near the area of the start codon are more likely to be active has yet to be substantiated by systematic studies. The seemingly disproportionate representation of such ASOs in the literature could easily be accounted for by the ubiquity of that particular strategy rather than any advantages that it may confer.

When ultimately deciding how to approach ASO design, it is worthwhile to investigate the services of commercial vendors. Considering the potential losses in productivity (it may divert a significant proportion of time from other projects), the ensuing distraction from the real biological questions and the expense of developing a reliable screening assay, the commercial price tags may seem more reasonable. In some cases, commercial vendors also offer access to proprietary DNA chemical modifications which may be advantageous.

5.2. Antisense selectivity

Next to the identification of a biologically active ASO, sequence selectivity is the second most important factor to be considered in the design and interpretation of antisense studies. Part of the allure of antisense comes from claims of absolute selectivity, yet there is surprisingly little evidence to support this contention. On the contrary, evidence exists which presents considerable challenges to this assumption. Woolf et al. studied the effect of mismatches (substitution of bases that are not complementary to the target RNA) on the activity of ASOs in *Xenopus* oocytes [112]. Whereas an intact 25-mer produced 79% degradation of the target mRNA, sequences with 17/25 and 14/25 matches resulted in 32 and 37% degradation, respectively. An unrelated control produced 13% degradation. Thus, mismatch of nearly half the sequence failed to completely eliminate ASO activity. The same study also concluded that as few as 10 consecutive complementary bases are sufficient to produce cleavage of a target RNA and that this is not prevented by mismatched flanking sequences. Thus, any 10-base stretch within an ASO could hybridize to any complementary 10-base stretch of RNA and produce non-selective effects. The implications of this study regarding the selectivity of antisense treatment are alarming. Hypothetically, assume that a 20-mer generally requires 6/20 base switches for complete loss of efficacy (a number commonly found in the literature). One must, therefore, also assume that a given 20-mer could potentially hybridize to and block the expression of any mRNA with which it has 15 or more matches. Add to that the fact that any given 20-mer will contain 11 10-mers, each capable of binding to complementary RNA and non-selective antisense effects on genes that may or may not be related to the targeted gene become a very real possibility. Whether this theoretical prediction presents grounds for concern in practice is difficult to assess at present. A thorough evaluation of potential non-selective antisense targeting would require a comparison of all possible combinations of (up to six) mismatches and 10-mers against the database of available sequences while taking into account RNA accessible sites. A further complication arises from the fact that the genome of the rat, the species of choice for many antisense studies, is not completely characterized. From a practical standpoint, the use of "gapmer" ASOs (see Section 5.3), where the length of the RNase H-sensitive sequence is limited, can potentially reduce the risks of non-selective effects.

Based on current standards, an ASO is generally considered to be selective if (a) it produces a functional effect, (b) mRNA or protein knockdown of the target is demonstrated and (c) control ONs have no effect on function or expression levels. This case is considered to be strengthened by the demonstration that the expression and/or function of a completely unrelated gene is unaffected, but this observation only provides support for a lack of general toxicity or disruption of RNA synthesis following ON administration. In some cases it may not be possible to selectively target a specific subtype within a closely related family if it has limited unique regions and these regions are not sensitive to ASO treatment. In such cases, however, it may be possible to target a subset of the family members (i.e., subunit 1 and 2 but not 3) or to develop a pan-antisense which targets the entire family (for example, see Ref. [31]).

In addition to inadvertent non-selective targeting due to sequence homology, knockdown of a target may be associated with changes in the expression, trafficking or function of other related or unrelated genes due to mechanisms such as co-regulation. This may result in assigning to the target functional properties that should be attributed to "non-selectively" affected genes. It is therefore important that the demonstration of knockdown of the target is accompanied at a minimum by evaluation of closely related genes. At present, the vast majority of

published papers fail to provide any evidence that closely related family members are unaltered by ASO treatment. This is particularly problematic in cases where the family members are known to have similar function and the goal of the study is to isolate the function of a single member from its close relatives [50,113–115]. The number of members in the family and/or the lack of appropriate tools may impose practical limitations on the number of relatives that can be examined. However, the identity of the family members most likely to complicate interpretation can be narrowed down significantly by taking into account what is known about their anatomical distribution and function. The Na^+ channels NaV1.8 and NaV1.9, for example, are the only TTX-r subunits expressed in sensory neurons [116] and upregulation of TTX-r currents in injured nerves contributes to their hyperexcitability [16]. Therefore, the demonstration that ASOs targeting NaV1.8 but not NaV1.9 had functional consequences in neuropathic pain models [20] suggests that the injury-related changes in TTX-r channels can be attributed to NaV1.8. In addition, the expression of two TTX-sensitive relatives was not altered by NaV1.8 ASO treatment [20]. Although a stronger case could have been made if NaV1.9 protein levels were also shown to be unaffected by ASOs targeting NaV1.8, the within family controls described above greatly substantiate the interpretation of the findings.

The level of complexity increases when considering the consequences of targeting a member of a protein family that forms functional heteromers. The loss of a heteromeric "partner" could have profound impact on the remaining partners, causing changes in expression, trafficking and subunit composition [117–119]. In the P2X family of ion channels, subunits P2X2 and P2X3 are known to form heteromers in sensory neurons. P2X3 homomers are also found in these cells but P2X2 homomers are not [2]. The role of the P2X3 subunit in chronic pain has been evaluated in both antisense and knockout studies. Changes in the expression level of the P2X2 subunit were not evaluated in any of these studies [10–13]. Without controlling for changes in P2X2 protein expression, many of the findings presented in these reports could have alternative explanations. For example, if P2X2 requires P2X3 for proper trafficking to axon terminals where it produces a functional effect, then it may be incorrect to attribute all functional changes directly to P2X3. Furthermore, loss of a subunit may cause formation of aberrant homomeric or heteromeric channels. It has been shown for both the $GABA_A$ and the NMDA receptors that ASO-mediated knockdown of certain subunits modulates the composition and properties of the remaining channels [117,118,120,121]. The resultant functional differences could therefore be due to the appearance of inappropriate channels rather than to the loss of a single subunit.

The adequate evaluation of antisense selectivity has been hampered in part by the daunting magnitude of performing expression analysis of a large number of proteins. Recently developed approaches for large-scale expression profiling at the RNA and protein level offer the opportunity for comprehensive and efficient evaluation of the global effects of antisense treatment [122,123]. DNA microarray studies have reported ASO-mediated changes in the expression of tens to hundreds of genes in addition to the targeted transcript [124,125]. These changes were attributed to hybridization of the ASO to homologous sites [124] as well as secondary effects on genes that may be functionally modulated by the targeted gene [125]. Understanding the global effects of antisense treatment is critical for its application as a functional genomics tool in drug discovery because they may impact the ability to predict the therapeutic potential of novel drug targets. Furthermore, the ability to analyze the global effects of ASO-mediated knockdown of pain-relevant proteins offers a novel application of ASOs to the study of pain and analgesia. For example, knockdown of a growth factor or a component of a second messenger pathway is likely to interfere with the expression of proteins regulated by the targeted gene. Therefore, large-scale analysis of protein expression following ASO treatment could provide information on signaling pathways downstream from the target gene.

5.3. Oligonucleotide chemistry

The use of unmodified phophodiester (PDE) ONs is generally not recommended. Their high susceptibility to nuclease degradation, resulting in a very short half-life, has limited their utility in most applications. The CNS, however, represents a special

Table 2
Examples of experimental design and outcome measures

Family (target)	Experimental design (chemistry, delivery, dose, controls)	In vivo assay(s)	Evaluation of knockdown	Ref.(s)
Ion channels				
P2X (P2X3)	2′-MOE gapmer with PDE or PS backbone Slow infusion (i.t.) 24–240 μg/day (rat) Universal or four-base MM	Spinal nerve ligation and sciatic nerve injury Peripheral inflammation Formalin α,β-ATP-hyperalgesia	~50%↓ mRNA in DRG ~20%↓ in staining by IHC in spinal cord ~35–45% ↓ in DRG by Western blot (range = 0–65%)	[12,13]
NaV (1.8, 1.9)	Phosphodiester (PDE) 2x daily injection (i.t) 90 μg/day (rat) Six-base MM	Spinal nerve ligation Peripheral inflammation Visceral (bladder) pain Na + currents in DRG and sciatic nerve	~45% ↓ in DRG by Western blot ~50–75% ↓ by IHC in DRG by cell count or intensity ↓ by IHC in nerve (qualitative)	[18–21]
NMDA (R1, R2C, R2D)	Phosphodiester (PDE) 2× daily injection (i.t.) 10 nmol/injection (rat) Sense & scrambled	Formalin	20–40% ↓ in NMDAR1, ~30% ↓ in NMDAR2C; ~40% ↓ in NMDAR2D of mRNA by RNase protection in spinal cord	[23]
5HT ($5HT_3$)	Phosphodiester (PDE) One injection per 48 h (i.t.) 20 μg/injection (mouse) Four-base MM	5-HT-agonist induced analgesia	$5HT_3$ binding was undetectable in ASO-treated group	[24]
G-Protein coupled receptors*				
α_2-Adrenergic receptors (α_{2A}AR, α_{2C}AR)	Phosphodiester (PDE) 2× daily injection (i.t.) 15 nmol/injection (rat, cervical) 12.5 μg/injection (mouse) Sense, four-base MM	α_2AR agonist-induced inhibition of NMDA or substance-P evoked behaviors	25–50% ↓ by IHC in spinal cord	[47,50]

Table 2. Continued

Family (target)	Experimental design (chemistry, delivery, dose, controls)	In vivo assay(s)	Evaluation of knockdown	Ref.(s)
Metabotropic glutamate receptors (mGluR1)	PS-endcapped PDE Slow infusion (i.t.) 6–50 μg/day (rat) Sense, four-base MM	Acute pain—tail flick & dorsal horn activity CFA-induced inflammation Sciatic nerve injury Morphine tolerance	15–35% ↓ by IHC Large range by Western blot analysis (~30–100%)	[51–54]
Galanin receptors (GalR1)	Peptide nucleic acids 2× daily injection (i.t.) 1.5 nmol/injection (rat) Scrambled	C-fiber-induced facilitation of nociceptive transmission	26% ↓ in Galanin binding by autoradiography	[55,56]
5-HT receptors (5-HT_{1B})	Phosphodiester (PDE) One injection per 48 h (i.t.) 20 μg/injection (mouse) Four-base MM	5-HT-agonist induced analgesia	70% ↓ in binding	[24]
Miscellaneous				
nNOS	Phosphodiester (PDE) One injection per 48 h (i.c.v.) 5–20 μg/injection (mice) Four-base MM	Morphine tolerance Morphine analgesia	~50–80% ↓ by mRNA ~35% ↓ in enzymatic activity	[76]
β-Arrestin	Phosphodiester (PDE) 2× daily injection (i.t.) 5 μl of 2 nM/injection (rat) Four-base MM	Morphine tolerance Nerve injury-induced allodynia	60% ↓ in mRNA (compared to MM control)	[75]
Neurotrophins	Fully phophorothioated Slow infusion (i.t.) 0.6 nmol/day (rat) Sense controls	Nerve-injury induced sprouting & allodynia	~45%↓ in spinal cord by ELISA	[77]

* ORs are not included in the table due to the volume of relevant publications.

case in which unmodified PDEs are stable enough to produce antisense effects. This is perhaps due to reduced nuclease activity in the CNS compared to peripheral tissues and serum. However, even in the CNS it is often necessary to deliver PDEs by continuous or repeated dosing to maintain active concentrations. Significant efforts have been made towards the development of modified ONs with increased stability, enhanced RNA binding affinity and reduced toxicity. Some issues to consider when evaluating the suitability of a particular chemical modification include binding affinity, stability, toxicity, uptake, mechanism of action, cost and availability. Of the hundreds of modifications that have been developed, those with the most relevance to the CNS are discussed below.

3′-Inverted

One approach to increase ON stability has been to invert the polarity of the terminal bases [83]. The assumption is that the 3′-inverted end would be less susceptible to exonuclease-mediated ASO degradation. The benefits of this modification have not been conclusively demonstrated.

Phophorothioates

The development of ONs in which the PDE bonds between nucleotide bases are modified to form phosphorothioate bonds has resulted in ONs with increased resistance to enzyme degradation while retaining RNase H induction. Often referred to as PS-ONs, phosphorothioate ONs have been very useful in in vitro systems and have been successfully applied in many in vivo studies.

PS-ONs are more potent than PDE-ONs in the CNS [83,126]. In a comparative study PS-ONs and 3′-inverted PDE-ONs targeting the corticotrophin releasing factor type-2 receptor were administered intracerebroventricularly (i.c.v.). Whereas a single injection of 0.5 nmol resulted in a 50% reduction in CRF binding, PO-ONs needed to be given twice daily at a 10-fold higher dose to produce a 42% decrease. Unfortunately, PS-ONs suffer from a number of serious limitations. They are notorious for having multiple non-antisense effects, probably due to their documented binding to many extracellular, cell surface and intracellular proteins [79]. To further complicate matters, these non-antisense effects may exhibit some sequence dependence, which can therefore easily be mistaken for biological effects one might expect from inhibiting the target mRNA. The most serious problem, however, is their toxicity in CNS. For example, i.c.v. administration of PS-ONs results in fever, weight loss and various symptoms of ill health [83,127,128]. These effects are independent of nucleotide sequence but are dose-dependent. In addition, intraparenchymal delivery of high doses of PS-ONs results in severe tissue damage characterized by cell loss and the appearance of lymphocyte-like cells in the damaged region [126]. PS-ON use in the CNS is therefore contraindicated.

Endcap PDE-PS chimeras

In an attempt to maintain some of the beneficial qualities of PS-ONs while reducing toxicity, chimeric ONs in which the first and last few bases are linked by PS bonds while the rest of the backbone is PDE have been tested. The rationale for "endcapping" is that the terminal PS linkages confer resistance to exonuclease activity. Endcap ONs exhibit similar efficacy to fully phosphorothioated ONs with reduced toxicity, although they do result in some cell damage [126]. In a related approach, the middle eight bases of a PDE-ON were replaced with PS linkages to protect the ASO against endonuclease activity. These chimeras were well tolerated for up to 9 days and demonstrated significant efficacy [83]. In another study, both endcap ONs and a 15-mer with five PS residues in the center showed signs of toxicity following intraparenchymal injection, albeit less severe than fully phophorothioated ONs [129]. Thus, although PDE-PS chimeras have higher efficacy than PDEs and are less toxic than PS-ONs, the toxic effects limit their utility.

Peptide nucleic acids (PNAs)

PNAs are ON analogues in which the deoxyribose phosphate backbone is replaced by a neutral polyamide backbone. They are resistant to nuclease activity and form more stable DNA/DNA and DNA/RNA complexes [130]. Initially it was thought that the utility of PNAs would be limited by poor cellular uptake. Efforts were therefore made to couple them to transporter peptides to enhance uptake. This approach was used successfully to decrease expression of the galanin receptor GalR1 following in-

trathecal (i.t.) delivery of PNAs [55]. It was later shown, however, that PNAs could be delivered intracellularly to neurons without transporter peptides following direct injection into the PAG [62] and that i.t. administered unmodified PNAs could inhibit GalR1 without uptake enhancers [56]. Thus, uptake into neurons did not appear to be a limitation of PNAs. Furthermore, it was demonstrated that systemically (10 mg/kg; i.p.) administered PNAs could cross the blood brain barrier and reduce gene expression in the PAG [63]. However, in another study using a transgenic mouse model in which ASOs correct aberrant splicing of EGFP (enhanced green fluorescence protein), systemically administered PNA-ASOs did not have a CNS effect [131]. Finally, PNAs are not substrates for RNase H and their effects may be more complex than just knockdown of the targeted gene. For example, PNA ASOs have been shown to alter the splicing of the interleukin-5 receptor-alpha chain; the expression of the membrane-bound isoform was inhibited whereas that of the soluble one was enhanced [85].

Locked nucleic acids (LNAs)

LNAs are ribonucleotides in which the 2′-oxygen of ribose is connected to the 4′-carbon by a methylene bridge. LNAs dramatically increase the stability and binding affinity of ASOs [132]. The melting temperature of an unmodified PDE ASO targeting DOR1 increased from 59 °C to >90 °C when fully LNA-modified [129]. Although fully LNA-modified ASOs do not activate RNase H, LNA:DNA:LNA gapmers (see below) are potent activators of RNase H. LNAs have been delivered to the CNS by i.t., i.c.v. and intrastriatal administration [129]. Spinally delivered LNA:DNA mixmers and gapmers were found to be more potent than unmodified PDE-ONs and fully-LNA modified ONs showed no signs of toxicity following intraparenchymal administration [129].

Gapmers

Gapmers are ONs which consist of a PDE or PS center flanked by modified nucleotides. The modified ends of the gapmers result in increased stability and/or RNA affinity while the stretch of PDE or PS monomers in the center confers ability to activate RNase H. Chemical modifications that have been used for gapmer design include LNA, PNA, and replacement of the hydrogen at the 2′-position of ribose by a methyl (2′-OMe) or methoxyethyl (2′-MOE) group [132,133]. Whereas a center stretch of seven or eight PS monomers are required for LNA:DNA activation of RNase H, 2′-OMe gapmers require only six [132]. To date there is no evidence of gapmer-mediated toxicity. LNA and 2′-MOE gapmers have been used in vivo in pain-related studies [12,13,129]. Although the 2′-MOE modification is not available for general use, the related 2′-OMe modification is commercially available and 2′-OMe gapmers have been shown to target successfully the capsaicin receptor VR1 in vitro [132].

5.4. Controls

Controls are a crucial component of any experimental design. In antisense studies, inactive DNA sequences are used to control for the specificity of the treatment. There is currently little to no agreement regarding the most appropriate DNA controls. It is well known that ON administration may have non-specific effects in addition to the desired antisense-mediated knockdown in expression of the target gene. Many of these effects are difficult to predict and/or explain given our current knowledge and are highly dependent on the specifics of each experiment (e.g., chemistry, route of administration, dose).

Administration of ONs to the CNS, regardless of sequence, may result in increased body temperature, reduced fluid intake, weight loss, decreased motor activity, changes in nociceptive thresholds, neuronal loss, induction of interleukin-6 and infiltration of lymphocyte-like cells as well as other signs of general sickliness [83,126,129,134,135]. The nature and intensity of these effects varies with many factors such as dose, route and duration of administration and ON chemistry. The inclusion of the proper controls is therefore essential for the interpretation of antisense experiments.

Oligonucleotide effects can be sequence-dependent, sequence-related and sequence-independent [136]. Sequence-dependent effects include hybridization to the target mRNA and the inadvertent targeting of homologous mRNAs. Sequence-related effects do not have an antisense-based mechanism but are

associated with specific DNA motifs and/or the formation of secondary or higher-order structures, such as guanosine-quartets, CpG motifs or palindromic sequences [93,109,137]. Sequence-independent effects arise from the chemical properties of the ONs. The use of the term non-specific effects often encompasses all effects of an ON not attributable to an antisense-based action at the target gene. However, since sequence-dependent and sequence-related effects may have a specific mechanism of action, such as affinity for a related mRNA, the term non-specific will only be used here to describe sequence-independent effects. It should be noted that the contribution of sequence-independent effects to the observed effects of ASO-treatment can only be adequately evaluated based on comparison of the DNA control group to a vehicle control group. The importance of vehicle controls is further discussed in Section 5.7.

Many types of DNA controls have been used in

Table 3
Advantages and disadvantages of DNA controls

	Advantages	Disadvantages
Mismatch	Can perform mismatch vs. function studies May control for problematic motifs Control for physical properties such as size and base composition	Potential residual hybridization at target Potential hybridization at non-target genes Possible appearance of problematic motifs Results may be difficult to interpret Difficult to design
Scrambled	Will not hybridize with target mRNA Control for physical properties such as size and base composition	Potential hybridization at non-target genes Appearance of and/or failure to control for problematic motifs Difficult to design
Reverse	Will not hybridize with target mRNA May control for some problematic motifs (if not orientation sensitive) Control for physical properties such as size and base composition Easy to design	Potential hybridization at other genes Appearance of and/or failure to control for problematic motifs
Sense	Easy to design	Potential hybridization at other genes High incidence of unexplained biological activity Appearance of and/or failure to control for problematic motifs
Universal	No design necessary Excellent control for sequence-independent effects	Not well represented in literature Failure to control for problematic motifs and most other physical properties such as base composition Potential for confounding effects unexplored
Targeting of unexpressed or unrelated genes	Control for sequence-independent effects	Failure to control for problematic motifs and most other physical properties such as base composition An "unrelated" or "unexpressed" gene may turn out to be "related" and/or "expressed"
Non-expressing cells	Useful in vitro to demonstrate selectivity of ASO	Not useful in vivo
Additional ASO sequences	Provides multiple converging lines of support for experimental findings	Increased use of resources Difficult to find multiple unique active sequences against a target gene
Isosequential modified ASOs as controls	Control for non-specific effects	The use of additional chemistries may require changes in protocols and could become very labor intensive

antisense studies and each one has its own peculiar share of advantages and disadvantages. It is therefore important when designing or evaluating antisense studies to be cognizant of the limitations of the different types of DNA controls. In general, DNA controls should be of the same length and chemistry as the ASO and should lack homology to other known genes. In addition, motifs such as G-quartets must be equally present or absent in both antisense and control ONs. Table 3 and the discussion below highlight some of the key issues to consider for the commonly used DNA controls.

Mismatch oligonucleotides as controls

Mismatch (MM) DNA controls, in which several pairs of bases within the antisense sequence have been swapped, are common in the literature. The primary advantage of MM controls is the retention of many properties of the active ONs such as molecular weight and GC content. In addition, MM ONs may retain problematic sequence motifs and higher-order structures characteristic of the ASO sequence. Thus, both sequence-related and sequence-independent effects may be accounted for.

The main disadvantage of MM controls is the potential for residual activity at the target gene, which can compromise interpretation of the results. If, for example, an experiment is performed in which a MM control produces a partial effect, it would not be possible to distinguish between the following possibilities: (1) the partial effects are due to residual affinity at the gene of interest (sequence-dependent). (2) The partial effects are due to the inadvertent targeting of a related or unrelated gene (sequence-dependent). (3) The partial effects are due to a specific motif that may or may not be present in the ASO sequence (sequence-related). (4) The partial effects are due to general toxicity of the compound itself (sequence-independent).

The potential for residual hybridization at the target gene raises the question of how many bases need to be switched to eliminate target-specific hybridization. As discussed above in Section 5.2, it has been suggested that mismatch of nearly half the sequence may fail to eliminate the ASO activity [112]. In another study a mRNA was targeted by four different active ASOs, each with their own mismatch controls. While all four ASOs inhibited mRNA levels, two of the MM controls had partial efficacy and two lacked efficacy, highlighting the variable nature of MM controls. The number of switches necessary to eliminate hybridization is currently theoretically unpredictable and will depend on many complex factors, including the affinity of individual ASO for their RNA binding site, the location of the mismatches (mismatches at the 5′ or 3′ ends are less effective than those in the middle), the type of base (some substitutions will have greater effects on hybridization affinity than others) and the total number of bases swapped.

In light of the limitations described above, the use of MM ONs as controls is therefore not advisable. In the event that it is necessary to use MM ONs to account for a problematic motif, examination of a series of MM ONs with increasing numbers of switches should ideally be employed. A correlation between the number of misplaced bases and loss of function strongly supports a sequence-specific mechanism of action for the ASO [138,139]. Alternative approaches include generating multiple MM controls, in which independent base pairs are swapped or combining MM with another type of control (i.e., Universal, see below). An unavoidable consequence of the above-mentioned strategies, however, is an increase in the expense of each experiment.

Scrambled oligonucleotides as controls

A variation of the mismatch approach is to design a control ON by completely scrambling the ASO sequence. Scrambled ON controls, in maintaining the GC content of the ASO, share many of the advantages of MM controls but are free of lingering questions regarding residual hybridization at the target or closely related genes. However, they do share some of the other disadvantages of MM controls such as the unpredicted inadvertent hybridization to unrelated genes. A limitation of scrambled controls is the failure to control for problematic motifs that may be present in the ASO such as G-quartets.

Reverse oligonucleotides as controls

Reverse ONs are prepared by synthesizing an ASO sequence in reverse. The reverse control for an ASO reading 5′-ATCCG-3′ would be 5′-GCCTA-3′. Reverse controls share the same advantages and

disadvantages with scrambled controls but are easier to design. However, since the sequence is predefined by the ASO sequence, the use of reverse controls is limited by their potential homology to other genes.

Sense oligonucleotides as controls

Sense controls are those complementary to the ASO and identical to the mRNA target. The sense control for an ASO reading 5′-ATCCG-3′ would therefore be 5′-CGGAT-3′. There are many disadvantages to their use including the failure to control for problematic motifs (i.e., GGGG becomes CCCC). There is a prevailing view that sense controls produce biological effects at a higher frequency than can be attributed to sequence-independent effects alone [136]. Despite the lack of hard data supporting this notion, sense controls have little advantage over other control sequences and therefore can be avoided.

Universal or "randomer" oligonucleotides as controls

Universal control DNA consists of a mixture in which the probability of an A, T, G or C being inserted at each position is equal. The result is a mixture of low concentrations of all possible sequences. This mixture is an excellent control for non-specific effects such as DNA toxicity. Other advantages include ease of design and the ability to control for multiple antisense sequences simultaneously. However, our lack of knowledge regarding the potential effects of low levels of DNA hybridizing to many mRNAs simultaneously is a cause for concern.

Antisense targeting unexpressed or unrelated genes as controls

The inclusion of an ASO known to be active against a gene not expressed in the experimental system can be used as a control (i.e., targeting luciferase in rat spinal cord). A negative result with an ASO that is active in other systems is considered by some to provide stronger support for selectivity than would a scrambled or reverse control. However, from the perspective of the spinal cord, a sequence with activity at luciferase looks no different from scrambled except in its failure to control for GC content-related factors such as molecular weight or hybridization strength. These controls are therefore less advantageous than scrambled or reverse. In an extension of this strategy, genes that are expressed in the experimental system but are thought not to be involved in the phenomenon under investigation could be used as controls. In the event that knockdown of the unrelated gene is validated yet has no functional effect in the experimental assay, specificity will have been well demonstrated. However, identification of a candidate "inert" gene is problematic and in the event that the control has partial efficacy the experiment would be uninterpretable.

Non-expressing cells or knockouts as controls

One can test for non-specific effects of an ASO in a system where the target gene is not present. This is particularly applicable to in vitro systems in which the target gene is being artificially expressed. In such cases, the effect of ASO-treatment in untransfected cells can be compared to those expressing the target gene. Any additional effects observed in the transfected cells would be considered to be antisense-mediated and sequence specific. However, this experiment would not control for the potential emergence of undesired sequence-dependent effects in vivo. Consider, for example, a situation in which genes closely related to the target mRNA are expressed in vivo but not in the in vitro system. The potential for cross-reactivity between related genes will be missed in vitro. In addition, in vitro assays will not control for non-sequence dependent effects that might arise in vivo such as toxicity at the site of administration. There are currently no published reports of ASO-treatment in a knockout animal. Ideally, the antisense should have no activity compared to DNA controls since the target gene is absent. Any other result would be difficult to interpret.

Additional antisense sequences as controls

The ability to demonstrate similar effects with multiple ASOs targeting the same gene strongly supports an antisense-based mechanism of action *if and only if* the potential for non-specific effects has been convincingly eliminated with appropriate DNA controls.

Is sequential antisense oligonucleotides as controls

If the same ASO, synthesized with two different

chemistries, decreases expression of the target gene and produces a consistent biological phenotype in independent experiments, it would be considered highly unlikely that the ASO-mediated effects were non-specific. However, this type of experiment should be considered complementary to and therefore only used in addition to proper DNA controls.

5.5. Route of administration

ONs are unable to penetrate the blood–brain barrier and are delivered to the CNS either i.t., i.c.v. or intraparenchymally using chronic indwelling catheters. Due to limited diffusion [140–142], these approaches target a relatively small area in proximity to the catheter or cerebral ventricles. Another major disadvantage of these approaches is their invasiveness, which can result in non-specific effects. Behavioral assays are particularly sensitive to the stress and discomfort associated with invasive surgeries. Potential non-invasive alternatives for antisense administration to the CNS include intranasal delivery [143–145] and systemic delivery of stable chemically-modified ASO to the CNS [61,63,146,147]. Finally, transcutaneous administration may be useful for delivery to sensory neurons via their peripheral terminals [148].

Intrathecal delivery is the most common route of administration used in pain-related antisense studies. It is usually accomplished via a chronic indwelling catheter [149], although many groups employ direct lumbar puncture, particularly in mice [150]. The primary limitations of the direct lumbar puncture method for antisense studies are the need for repeated injections and the skill of the injector. Since the injector should be blind to the treatment groups and no clear functional readout for successful injection is immediately available, the injector must have demonstrated a consistently high ($>90\%$) success rate.

In rats and larger animals i.t. delivery is almost always accomplished using catheters. The exposed end of the catheter can be used for bolus injections or can be attached to an osmotic pump for slow infusion over time. A direct comparison between bolus injections and minipump delivery of ASOs noted significantly greater ASO uptake in dorsal root ganglia (DRG) cell bodies following slow infusion [13]. This method also requires less involvement of technical staff and causes less stress in the animals. There are two main approaches to catheter placement. The most commonly used is to extend the catheter from the cisterna magna to the rostral end of the lumbar enlargement. Although animals catheterized in this manner do not have overt pain symptoms, there are signs of compression injury to the spinal cord as well as formation of scar tissue around the end of the catheter [151]. A more recently developed method inserts a catheter at the level of the lumbar enlargement using direct lumbar puncture [152]. This method is preferable for both humane and scientific reasons but is currently more labor intensive.

The position of the catheter relative to the dorsal and ventral horns of the spinal cord varies considerably. Given the observation that dorsally placed catheters result in ASO effects in dorsal but not ventral horn [51], ventrally placed catheters are unlikely to result in knockdown of proteins expressed in the superficial dorsal horn. Therefore, inclusion of animals with ventrally or laterally placed catheters is likely to contribute to increased variability in pain-related antisense studies. The location of the catheter can be determined using lidocaine prior to functional analysis [54] or using post-mortem analysis [51] and only animals with dorsally placed catheters should be included in the analysis.

5.6. Oligonucleotide uptake

ASOs are readily taken up in the CNS without the aid of uptake enhancers. The presence of ASO in sensory neurons following i.t. administration has been visualized using fluorescent probes and antibodies recognizing chemical modifications [13,19]. However, the series of events that deliver the antisense from its point of infusion to the sensory neurons as well as the factors that influence these events are unclear. Although the extent of diffusion of ASOs within the i.t. space is uncharacterized, it appears that the ASO effect is restricted to the region caudal to the catheter [19]. The effect of the rate of infusion is unknown, but this factor is likely to influence the effective concentration of ASO at the site of action throughout the treatment. The precise site(s) of neuronal uptake of ASO after i.t. administration has not been determined; possibilities include the central terminals in dorsal horn, the dorsal roots

or the DRG cell bodies. Furthermore, the relative degree of ASO uptake in DRG neurons versus spinal cord neurons has not been characterized. However, uptake of fluorescently tagged ONs has been shown in spinal cord [22] and antisense has been used to successfully target proteins expressed in dorsal horn [23,50], indicating that ASOs have access to spinal cord neurons. From a practical standpoint, factors that should be considered when choosing a probe for the visualization of ASO delivery include the stability of the ON and the stability of the probe under the experimental conditions (e.g., structural integrity at body temperature, resistance to enzymatic cleavage, compatibility with fixation).

Comparison of the in vivo uptake efficiency of ASOs with different chemical modifications may help elucidate these mechanisms and optimize the experimental design of antisense studies. For example, in vitro it has been demonstrated that in the absence of uptake enhancement, cationic (e.g., PNAs with four lysines at the 3′ end) and neutral (morpholinos) ASOs cross the cell membrane more efficiently than negatively charged ASOs (2′-MOE) [153]. Increased uptake of PNAs containing 4 lysines at the 3′ end has also been demonstrated in vivo [131].

Systematic comparisons of technologies designed to enhance cellular uptake of DNA in vivo are needed to optimize ASO delivery. Liposomal carriers are commonly used in vitro to facilitate uptake and many of these preparations are commercially available. Due to differences in their properties, it may be necessary to test several sources before identifying the best preparation for each in vitro system [89]. Although it has been shown in at least one study that liposomes did not facilitate ON uptake in the CNS [22], comprehensive screening of different preparations would be required to identify a potentially useful one. Furthermore, given our incomplete understanding of the mechanisms of ON uptake in the CNS, it is not possible to predict if the use of liposomes will confer any advantage.

An interesting strategy for uptake enhancement is the conjugation of ONs to carriers that may facilitate endocytosis. The conjugation of ONs to cell penetrating peptides has been shown to enhance ON delivery to the nucleus in vitro [154]. The potential of these methods for in vivo delivery in the CNS, however, remains to be fully explored. Furthermore, there is evidence that these peptides contribute to non-specific effects [124]. In another approach, biotinylated ONs were bound to a transferrin receptor monoclonal antibody-streptavidin (mAb-SA) conjugate [146,147]. Upon binding to the antibody, the transferrin receptor underwent receptor mediated endocytosis across the blood brain barrier, delivering systemically administered ONs into the CNS [146,147]. An alternative strategy, which may contribute to increased uptake as well as cell-specific targeting, is to conjugate the ON to a ligand which binds to a receptor expressed by the targeted cell type, thus inducing endocytosis. In one example, ONs were covalently bound to a glycoprotein which caused receptor-mediated internalization in the target cells [155]. It should be noted that the benefits of increased uptake of carrier-ON conjugates may be compromised by intracellular trafficking such as trapping in endosomes, which would limit the access of ONs to their site of action [156].

5.7. Oligonucleotide dose

In the famous Indian fable "The Blind Man and the Elephant", a group of blind men were assembled and each was presented a part of an elephant: the head, ears, tusk, trunk, foot, back, tail or tuft, and then asked to describe what sort of thing an elephant is. In describing just the part of the animal that they had access to, their descriptions were all simultaneously partially correct and completely wrong. In pharmacology, conclusions based on analysis of a single dose point are analogous to the blind men's descriptions of the elephant: simultaneously partially correct and completely wrong. In the pain-relevant literature there are surprisingly few studies examining the effects of ON dose and dosing schedule in vivo. As a result, the impact of these factors on antisense treatment in the CNS is unknown. It is known, however, that the therapeutic window might be narrow and that the dose range and ideal dosing schedules will change with chemistry, route of administration, the target gene and the experimental endpoint.

The importance of examining the full dose range of ON effects is illustrated in the hypothetical experiment presented in Fig. 2 and Table 4. In this example, the effect of an ASO is compared to one of

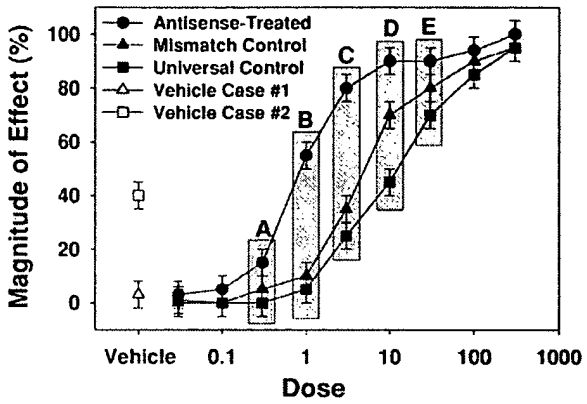

Fig. 2. Hypothetical data demonstrating the importance of dose–response analyses. The vertical grey bars delineate the % response at a single dose point. Depending on the dose examined, the results may lead to different conclusions, as outlined in Table 4. The results may also be interpreted differently depending on the response of a vehicle control group or in the absence of such a control.

two DNA control groups—a MM and a "universal" control. As shown in Table 3, this experiment would lead to fundamentally different conclusions depending on the presence of a vehicle control, the baseline set by a vehicle control and, most critically, the dose point examined. In the absence of a vehicle control group, evaluation of dose B or C would suggest the presence of an ASO-mediated effect whereas doses A and E would indicate a lack of effect in all ON-treated groups. In the presence of a vehicle control, examination of a single dose-point leads to an entire range of conclusions (no effect, ASO-specific effect, non-specific DNA effects) depending on the chosen dose.

Our current lack of appreciation for the importance of ASO dose is illustrated by examining the effects of i.t. ASOs targeting P2X3 in three independent studies. In one study, antisense and control ONs were synthesized as 2′-OMe gapmers with a PDE middle [157]. The 2′-OMe chemistry is related to the 2′-MOE chemistry used in the other two studies [12,13]. In all three studies ONs were administered by slow infusion through osmotic minipumps. The doses used and percent knockdown were as follows. (1) Doses of 2.4, 7.2 and 24 μg/day of 2′-OMe-PDE gapmers all resulted in an approximately 30% decrease in P2X3-immunoreactivity (P2X3-ir) by quantitative image analysis in the dorsal horn [157]. (2) Approximately 30% knockdown in P2X3-ir in dorsal horn and 50% in P2X3 mRNA measured by in situ hybridization in DRG was achieved using 2′-MOE-PDE gapmers at 180 μg/day [12]. (3) 2′-MOE-PS gapmers at 240 μg/

Table 4
Experimental inerpretations vary with dose and vehicle controls

	Dose	Antisense	Mismatch	Universal	Conclusion
Relative to DNA controls	A	–	–	–	Inactive ASO
	B	+	–	–	ASO-specific effect
	C	+	–	–	ASO-specific effect
	D	+	+ / –	–	ASO effect, non-specific DNA effect#
	E	–	–	–	Inactive ASO
Relative to vehicle control 1 (5% effect)	A	–	–	–	Inactive ASO
	B	+	–	–	ASO-specific effect
	C	+	+ / –	+ / –	ASO effect, non-specific DNA effect
	D	+	+ / –	+ / –	ASO effect, non-specific DNA effect
	E	+	+	+	Non-specific DNA effects
Relative to vehicle control 2 (40% effect)	A	(+)	(+)	(+)	Non-specific DNA effects*
	B	+	(+)	(+)	ASO effect, non-specific DNA effect*
	C	+	(+)	(+)	ASO effect, non-specific DNA effect*
	D	+	+ / –	–	ASO effect, non-specific DNA effect#
	E	+	+	+	Non-specific DNA effects*

These non-specific DNA effects are only observed in the MM control group.
* These non-specific DNA effects are bi-directional.

day produced an average of 40% knockdown by Western blot analysis [13]. The magnitude of P2X3 knockdown is therefore relatively constant from 2.4 and 240 μg/day. The apparent "saturation" of the knockdown could be rationalized in multiple ways, such as the relative efficacy of the sequences used and the possibility that intrinsic regulatory mechanisms limit the antisense effect to a maximum of 30–50%. Regardless, these data emphasize the need for systematic analysis of antisense effects in terms of dose dependency.

A final note about dose: the doses of ON delivered into the CNS are best expressed in terms of nmol and not μg. As the molecular weight of ONs changes with base composition, length and chemical modification, two samples, each with the same number of μg per unit volume, might have significantly different ON concentrations. Therefore, it would be technically incorrect to assume that groups of animals treated with equal volumes of these samples have been exposed to the same dose. When converted to nmol per unit volume, the concentrations are standardized across samples and direct comparisons can be made.

5.8. Dosing schedule

The level of protein expression and the rate of turnover will impact the efficacy, time course and dose requirement of antisense treatment. The longer the half-life of the protein, longer time–courses of administration may be required. For example, functionally relevant knockdown of G-protein α-subunits and β-arrestin can be achieved with single ASO injections [67,75]. In most protocols targeting GPCRs, animals are dosed for up to 3 days yet the half-lives are thought to range from 2 to 8 days [83]. Longer treatment periods may result in significant increases in knockdown. When ASO treatment against CRF_2R was extended from 5 to 9 days, the reduction in receptor binding increased from 40 to 60% to 80% [83].

In addition to the total length of treatment, the treatment interval chosen could have dramatic consequences on the results of a study. Fig. 3 depicts the effect of three different dosing schedules (12, 24 and 48 h intervals) on a hypothetical endpoint. The arrows indicate each time compound is administered. In this example, if the experimental endpoint is measured 12 h after the first treatment, similar effects would be observed in all three groups. If the first assay point is 24 h after the beginning of treatment, an effect will be observed in the 12 h group but not the others. If the measurements are taken 36 h following initiation then efficacy will be observed in the 12 and 24 h interval groups but not the 48. The purpose of this illustration is to stress the importance of the time-course of action following administration. In practice it may therefore be advantageous to give small doses several times daily or slow infusion rather than expose the animals to larger doses less frequently. This should reduce acute toxicity and maintain ASO concentrations at a more steady level. Furthermore, ASOs delivered i.t. by slow infusion have been shown to accumulate to much greater extent in DRG cell bodies than following repeated bolus injection [13].

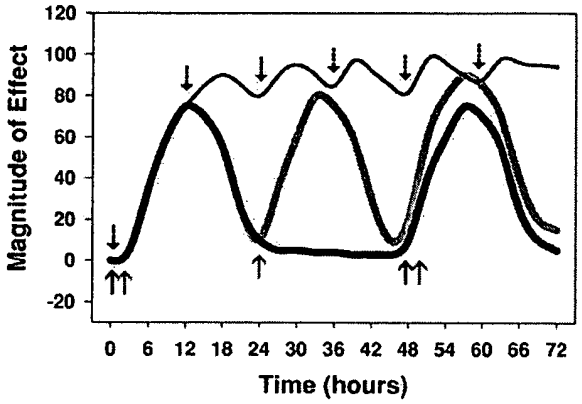

Fig. 3. Illustration of the effect that different dosing schedules may have on antisense efficacy. Each arrow represents an administration. The drug is administered every 48 (thick black; ↑), 24 (dark grey; ↑) or 12 h (thin black; ↓). Larger treatment intervals may introduce substantial fluctuations in the magnitude of the ASO-mediated effects. As a result, the effect of ASO-treatment may be missed if the experimental endpoint is not measured at the optimal time.

5.9. Evaluation of knockdown

The demonstration of antisense-mediated knockdown of the targeted gene is necessary for the interpretation of antisense studies. In the presence of knockdown, lack of a functional effect after antisense treatment would be interpreted as evidence that the targeted gene has no functional role in the

studied phenomenon. However, in the absence of knockdown, the lack of functional effect would be attributed to insufficient biological activity of the ASO and would provide no information about the function of the target gene. Similarly, without demonstration of knockdown, any functional changes observed after ASO-treatment cannot be attributed to ASO-mediated effect at the target gene.

In many antisense studies in the literature the magnitude of the functional changes exceeds that of the change in expression. For example, a 35–55% reduction in deltorphin II binding was associated with 80–90% reduction in deltorphin II-mediated analgesia [129]. Similarly, reduction of P2X3 expression by less than 50% resulted in the complete loss of αβmeATP-induced mechanical hyperalgesia and greater than a 50% decrease in αβmeATP-induced flinching behaviors [12,13]. This discrepancy is most likely associated with phenomena such as spare receptors and with the fact that the functional assay and the expression analysis may measure different pools of the targeted gene.

In addition to its importance for interpretation of functional results, systematic analysis of knockdown performed prior to functional analysis can be very beneficial in the design of the functional portion of the study. Information on the dose-dependence and time–course of changes in expression of the targeted gene provides a basis for selecting an appropriate dose range and dosing schedule for functional studies. It could be argued that performing expression analysis prior to functional analysis is not an efficient use of time and resources. However, expression analysis requires less animals per experimental group than functional studies. In the long run this approach may therefore save time and resources that would have been wasted using inactive ASOs or performing functional analysis at suboptimal dose and/or time-points.

The issues surrounding controls, dose and time–course discussed in the preceding sections are as relevant to the analysis of knockdown as they are to the functional analysis. In a number of studies the control MM ASO resulted in increased variability in the expression levels of the targeted gene compared to other experimental treatments (for example, see Refs. [19,54]), consistent with the possibility of residual efficacy of the MM. However, in the absence of other DNA controls it is impossible to determine if MM effects are due to residual efficacy at the target gene or to non-specific effects. Moreover, in some of these studies statistical significance is reported based on comparison of the antisense group to the vehicle group using a *t*-test, without comparison between the antisense and the mismatch group. In order to conclusively demonstrate an antisense-mediated effect, it is necessary to demonstrate that the antisense group is different from both the vehicle and the control DNA group(s) using statistical tests for multiple comparisons (for example, see Ref. [23]). Finally, there have been surprisingly few studies exploring the dose-dependence and time–course of antisense-mediated knockdown (for examples, see Refs. [71,158]). A correlation between the degree of knockdown and the functional effects across dose and/or time would support a causative link between them.

Antisense-mediated knockdown can be evaluated at the mRNA or the protein level, and ultimately the approach chosen is determined largely by the available tools and expertise. Levels of mRNA can be assayed by a variety of methods, including Northern blot analysis, in situ hybridization, RNA protection assay or real-time reverse transcription polymerase chain reaction (RT-PCR). Many antisense studies assess knockdown solely based on mRNA measurement (for example, see Ref. [23]). However, antisense treatment reduces mRNA levels only if the ASO acts through an RNase H-dependent mechanism, and therefore limiting the expression analysis to mRNA levels may result in a false negative. In fact, ASOs that act through non-RNase H-dependent mechanisms may even enhance the levels of the targeted gene [84,159]. Furthermore, the relationship between mRNA level and protein expression is often non-linear [122]. Given these difficulties, relying on mRNA assays to validate knockdown could lead to misinterpretation of the experimental results.

Ideally a comprehensive antisense study would include expression analysis at both the mRNA and the protein level (for example, see Refs. [12,126,160]). If this is not feasible, it is preferable to demonstrate decreases in protein levels because they will ultimately determine the levels of functional activity. The majority of methods for analysis of protein expression are antibody-based, including

immunohistochemistry (IHC) (for example, see Refs. [19,161]), Western blot analysis (for example, see Refs. [13,71,160]), enzyme-linked immunosorbent assay (ELISA) (for example, see Ref. [77]) and radioimmunoassay (RIA) (for example, see Ref. [126]). In all of these approaches, the quality of the results will first and foremost be limited by the quality of the antibody used. Non-antibody based methods such as receptor binding (for example, see Refs. [24,162,163]), autoradiography (for example, see Refs. [22,56]), or mass spectrometry are also available.

Evaluation of knockdown requires some form of quantitative analysis. Qualitative judgments based on visual observations are generally not accepted as proof of antisense-mediated effect. Based on the type of tissue processing, the methods for expression analysis fall into two major categories: histological (e.g., in situ hybridization, IHC, receptor autoradiography) and biochemical (e.g., Northern blot, Western blot, ELISA, RNA protection assay, real-time RT-PCR, mass spectrometry). Histological approaches offer the advantage of focusing the analysis on the relevant area. However, the quantification of histological observations relies on image analysis, which is not very sensitive, can be subjective and may be confounded by high variability due to tissue processing. Quantitative image analysis inevitably requires selection of a labeling threshold, which distinguishes specific signal from background. Although it is sometimes possible to establish a protocol for objective selection of threshold [164], it is more common for the observer to set the threshold subjectively based on the labeling in control groups. Strategies for minimizing the effects of variability and subjectivity include: (1) process samples from different experimental groups in parallel so that each group is represented on a given slide; (2) determine the threshold based on the labeling in the vehicle control group; (3) average measurements from several slides to obtain the final values for each subject; (4) perform the analysis blind to the experimental conditions.

Unlike histology, the tissue collection protocols associated with biochemical methods do not usually allow for precise microdissection of the region most likely to contain changes in expression. This could result in the dilution of any antisense effect, making it harder to observe changes. However, with the exception of methods based on electrophoresis and densitometry (Northern blot and Western blot), this disadvantage is outweighed by the superior sensitivity and, in some cases, the ability to measure absolute amounts in individual samples. Using an RNA protection assay, Yukhananov et al., determined that treatment with ASO targeting the NMDA2RD subunits decreased expression of the subunit from ~ 1.5 to ~ 0.75 amol/μg of total RNA (1 amol = 10^{-18} mol) [23]. A relatively new approach for RNA quantification is real-time RT-PCR, which measures mRNA levels based on the number of replication cycles necessary to detect the gene of interest in the sample relative to a housekeeping gene [165]. In its most commonly applied form, real-time RT-PCR measures are relative amounts, but methods for determining absolute amounts based on standard curves are under development [166]. This approach has been used for quantification of antisense-mediated knockdown [160,167] although due to its novelty, its representation is the literature is limited.

At the protein level, quantitative evaluation of knockdown by ELISA is particularly feasible in the case of neurotrophins [77], for which there are commercially available tools that have been applied in numerous experimental paradigms, including transgenic animals [168]. However, the measurement of absolute expression levels using RNA protection or ELISA requires the generation of a standard curve of known amounts of mRNA/protein, which is usually not trivial. An additional limitation in the use of ELISA is the requirement for high-quality antibodies. A more predominant method for quantification of knockdown at the protein level is receptor binding (for example, see Refs. [24,162,163]). It is sensitive as well as technically straightforward. However, just as ELISA is dependent on antibodies, receptor binding (and autoradiography) is limited by the quality and availability of ligands. Since the motivation for many antisense studies is the lack of selective ligands for functional distinction of subtypes, suitably selective ligands may not be available. The use of non-selective ligands for receptor binding is an option [56] although the presence of additional non-ASO-targeted binding sites will dilute the results, making it difficult to detect knockdown.

Advances in proteomic analytical methods based

on mass spectrometry offer new approaches for knockdown evaluation [169–173]. The advantages of mass spectrometry for protein quantification are its superior sensitivity in the fmol range and the ability to assess protein levels directly, independent of a ligand or antibody. Until recently, the application of mass spectrometry to analysis of complex protein mixtures from biological systems has been restricted by the limited capacity of two-dimensional gel electrophoresis (the predominant separation strategy) for detection of low abundance and transmembrane proteins [174]. This limitation has been overcome by the introduction of multidimensional liquid chromatography as an alternative separation strategy [171–173]. In addition, the use of isotope-coded affinity tags (ICATs) has allowed measurement of relative differences in protein levels between two samples by mass spectrometry [122,169,170]. Although the application of these methods to the mammalian nervous system is just beginning to be explored and is likely to be challenging, their adaptation to antisense studies may facilitate the analysis of ASO-mediated knockdown of protein expression.

6. Conclusions

The use of ASOs has contributed greatly to the field of pain and analgesia. Nevertheless, ASO-based approaches have failed to meet the high expectations initially placed upon them. This failure can be largely attributed to an underestimation of the complexity of antisense technology and a lack of appropriate tools for its successful application and evaluation. Over the past decade, sustained exploration has lead to an increasing understanding of the pitfalls encountered in in vivo ASO-based studies. This accumulated knowledge, in combination with continued technological developments, have set the stage for a long-overdue, systematic and in-depth analysis of ASO-mediated effects in vivo. Empirical and in silico methodologies for the selection of active sequences and the development of new chemistries have improved the design, specificity and stability of ASOs. Sensitive methods for the evaluation of ASO-mediated knockdown have enabled quantitative assessment of the dose- and time-dependency of action. As a result, our ability to design and interpret the results of functional studies has improved dramatically. Technologies for large-scale expression analysis at the RNA and protein level allow for global evaluation of both specific and non-specific ASO effects. Taken together, these advances have the potential to increase our understanding of the mechanisms of ASO-based approaches, facilitate the application of ASO as a functional genomics tool, and enable sophisticated approaches for the development of new pain therapies. The promise of antisense-based technology is therefore stronger than ever.

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Antisense-mediated redirection of mRNA splicing

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Abstract. Antisense technology has been used to study basic biological processes, and to block these processes when they deleteriously lead to human disease. A separate, equally important application of antisense technology is to upregulate the gene expression lost in the diseased state by shifting alternative splicing of pre-messenger RNA. This strategy has commonly relied upon the use of antisense oligonucleotides; however, another approach is to use a plasmid construct to generate antisense RNA inside the cell. Antisense therapeutics based on expression vectors and viral vectors offers a gene therapy approach, whereas those based on oligonucleotides offers a more drug like approach.

Key words. Antisense; splicing; oligonucleotides; snRNAs; gene therapy.

Alternative pre-mRNA splicing splicing

Alternative splicing is emerging as an extremely important process that mediates diversity in the proteomes of higher organisms by generating arrays of protein isoforms. In the most extreme examples, the slowpoke Ca^{++} ion channel [1] and the *Drosophila* gene dscam [2] can be potentially spliced into 500 and 38,000 isoforms, respectively. Since in humans 35–60% of genes are alternatively spliced, this process must account for a large fraction of the estimated 140,000 proteins present in human cells [3, 4].

The mechanism of alternative splicing is not well understood, as evidenced by the fact that attempts to predict legitimate splice sites in silico still generate substantial false positives/negatives [5–7]. While consensus sequences of the elements that control splice site selection, such as the 3′ and 5′ splice sites, the branch point and the polypyrimidine tract are well defined [8], under certain conditions sequences that only very loosely match the consensus sequences can be efficiently used [9]. More recent findings indicate that the function of the above elements may be modulated by exon and intron splicing enhancers and silencers, which contribute to the plasticity of pre-messengers RNA (mRNA) splicing [10]. As a result, splicing of pre-mRNA is not fixed, leading to alternative outcomes controlled by the cell. Importantly, this alternative splicing can also be controlled by external manipulation by antisense RNA and oligonucleotides.

Antisense RNA and oligonucleotides bind to the target pre-mRNA in a sequence-specific fashion, sterically blocking targeted splice sites and redirecting the spliceosome to available and unhindered splice sites. Thus, the antisense molecules act as silencers of the targeted splice sites and thus as enhancers of the alternatively selected sites. This application of antisense technology has clinical relevance, since many diseases are the result of misguided alternative or aberrant splicing [11, 12].

The role of splicing in disease

An annotated database survey showed that up to 15% of point mutations contributing to genetic diseases damage or modify splice sites and other sequence elements involved in splicing, and therefore result in aberrant splicing of pre-mRNA [10, 13]. This percentage could be much higher if it were based on RNA expression and splicing patterns rather than solely on genomic sequence. For example, when analyzed at the RNA level, 50% of

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mutations in the ataxia-telangiectasia and neurofibromatosis type 1 [14] genes resulted in defective splicing. Likewise, of the more than 900 sequence alterations detected in the cystic fibrosis transmembrane regulator (CFTR) gene, about 20% are splicing mutations [3]. Aberrant splicing may be caused not only by a defect in the existing splicing elements but also by mutations that create additional splice sites and/or activate cryptic splice sites. β Thalassemia, a hereditary blood disorder, provides a good example of this mechanism and will therefore be discussed in more detail below [15] and will therefore be discussed in more detail below. In addition, diseases caused by inappropriate splicing may be due not only to mutations in the given pre-mRNA, but also by factors that influence alternative splicing. For example, alternative splice site selection is changed in transformed cells, as demonstrated by the specific alternative splice variants of various pre-mRNAs expressed by many cancers [16].

Modification of splicing by antisense antisense RNA

Close to 200 mutations cause β thalassemia, but those that create additional, aberrant splice sites in introns 1 and 2 of the β-globin gene are among the most frequent worldwide [17]. This laboratory showed that blocking the aberrant splice sites with either antisense RNAs [18–21] or oligonucleotides [22–24] restores correct splicing and correct expression of β-globin and consequently hemoglobin in treated cells.

Figure 1 illustrates that mutations at nucleotides 654, 705 or 745 in intron 2 of the human β-globin gene activate aberrant 3′ and 5′ splice sites within the intron and prevent correct splicing of β-globin pre-mRNA, resulting in inhibition of β-globin synthesis and in consequence β-thalassemia. Treatment of the HeLa or K562 cells that stably expressed thalassemic genes with U7 and U1 small nuclear RNAs (snRNAs), modified to contain sequences antisense to the aberrant splice sites, resulted in reduction of the incorrect splicing of pre-mRNA and a concomitant

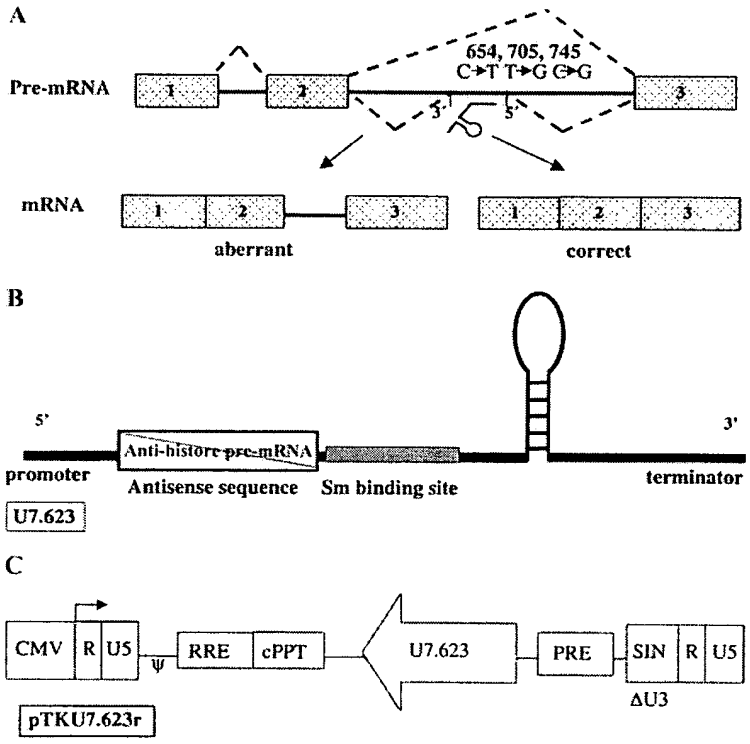

Figure 1. (*A*) Correction of splicing of β-globin pre-mRNA by modified snRNAs. Boxes, exons; lines, introns. The dashed lines represent correct and aberrant splicing pathways. (*B*) Structure of modified U7 snRNA constructs. Wild-type U7 snRNA includes a stem-loop structure, the U7-specific Sm sequence and a sequence antisense to the 3′ end of histone pre-mRNA. The promoter and 3′-terminator regions are indicated. In modified U7 snRNAs, the Sm and antisense sequences were replaced with the spliceosomal Sm sequence SmOPT and with antisense sequences targeted to the β-globin pre-mRNA. The SmOPT site is boxed and the antisense sequences are underlined. (*C*) Lentiviral vector design. The modified U7.623 snRNA was inserted between the central polypurine track of HIV-1 (cPPT) and the downstream long terminal repeat (LTR) of the pTK134 plasmid in reverse (pTKU7.623r) orientation. Transcription of the full-length vector RNA was driven by human cytomegalovirus (CMV) promoter. The vector also contains a packaging signal (ψ), the Rev response element (RRE), a sequence containing the woodchuck hepatitis virus post-transcriptional regulatory element (PRE) and a self-inactivating (SIN) deletion in the U3 region of the downstream LTR. Modified from [21].

increase in correct splicing, which ultimately led to increased levels of the correct β-globin protein [18–20]. This result demonstrates the advantages of targeting splicing: by definition, a decrease in one splice variant leads to an increase of its counterpart and results in a major change in the ratio of the products.

To apply this approach to the treatment of primary erythroid cells from thalassemic patients, with the goal of delivering the antisense RNAs to hematopoietic stem cells, the modified U7 snRNA gene was incorporated into a lentiviral vector. Delivery of this construct into HeLa cells expressing the three thalassemic mutants reduced the incorrect splicing of pre-mRNA and led to increased levels of the correctly spliced β-globin mRNA and protein [25]. Importantly, the therapeutic potential of this system was demonstrated in erythroid progenitor cells from a patient with IVS2-745/IVS2-1 thalassemia. Twelve days after transduction of the patient cells with the U7/antisense lentiviral vector, the levels of correctly spliced β-globin mRNA and hemoglobin A increased approximately 25-fold over background (fig. 2). Although this report did not provide direct evidence that hematopoietic stem cells were affected, similar vectors have been found effective in these cells [26]. Thus it is likely that this approach may have long-term effects, holding great promise for the amelioration of disease.

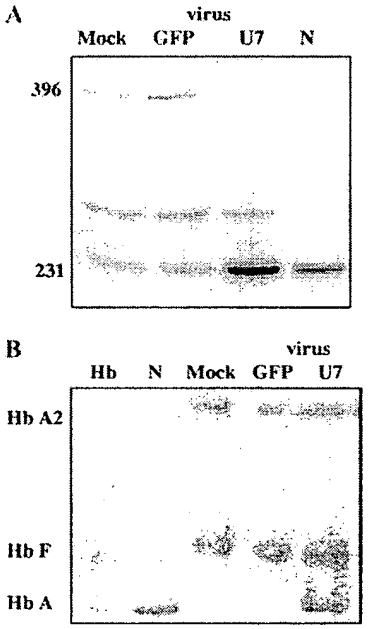

Figure 2. U7.623 lentiviral vector-induced β-globin pre-mRNA repair in erythroid progenitors from IVS2-745/IVS2-1 thalassemic patient. (*A*) Reverse transcription-polymerase chain reaction (RT-PCR). On days 1 and 2 of culture, the cells were transduced with either no virus (lane 1), control, green fluorescent protein (GFP) lentiviral vector (lane 2) or U7.623 lentiviral vector (lane 3). Lane 4, (N) RNA from normal blood. (*B*) Immunodetection of hemolysates separated by electrophoresis on cellulose acetate with anti-human hemoglobin antibody. Lane 1, hemoglobin standards; lane 2, (N) normal blood; lane 3, mock transduced cells; lane 4, cells transduced with GFP lentiviral vector, lane 5, cells transduced with U7.623 lentiviral vector. Modified from [21].

Antisense RNAs encoded in viral vectors have also been used to induce exon skipping in dystrophin pre-mRNA. Mutations that disrupt the dystrophin reading frame lead to premature termination of protein synthesis, and result in Duchenne muscular dystrophy (DMD). In contrast, mutations such as deletions and insertions that maintain the reading frame lead to the synthesis of shorter,defective but semifunctional dystrophin protein, and result in the less severe Becker muscular dystrophy (BMD) [27]. Therefore, if skipping the mutated exon removes the offending mutation and restores the reading frame it should be possible to convert the severe DMD phenotype to the BMD phenotype. This was accomplished by De Angelis et al. who replaced sequences in U1, U7 and U2 snRNAs with sequences antisense to the 5′ and 3′ splice sites of exon 51 in human dystrophin pre-mRNA [28]. These modified snRNA genes were then cloned into the 3′ long terminal repeat (LTR) of the pBabe puro retroviral vector. Viral particles were used to transduce muscle cells from a DMD patient having a deletion encompassing exons 48, 49 and 50. Since this deletion created a premature termination codon in exon 51, skipping this exon partially restored the reading frame of the gene and rescued dystrophin synthesis. The most efficient skipping was obtained when both the 5′ and 3′ splice sites of exon 51 were targeted with antisense molecules. These results indicate that a nonfunctional form of dystrophin mRNA can be converted into a functional form by antisense-induced shifting of splicing patterns.

Modification of gene expression by trans-splicing

Although most pre-mRNAs undergo cis-splicing to form mature mRNA, trans-splicing, whereby sequences from two independently transcribed pre-mRNAs are spliced to form a composite mRNA, also exists. Mansfield et al. [29] modified and enhanced this process, developing spliceosome-mediated RNA trans-splicing (SmaRT) technique. In this technique, fragments of pre-mRNA are designed to base-pair with the intron of a targeted pre-mRNA to enhance trans-splicing between the two molecules, while suppressing cis-splicing of the target. Using SmaRT, this group successfully corrected mutations in cells expressing the mutant form of the CFTR minigene, which contains a mutation (ΔF508) in exon 10. Mature CFTR protein was produced in treated cells, indicating that the newly generated mRNA was translated into correct protein. Recently, Liu et al. demonstrated that SmaRT could correct endogenous ΔF508 mutant CFTR pre-mRNA in cystic fibrosis (CF) airway epithelia and partially restore CFTR-medi-

ated chloride transport. An adenoviral vector was engineered to carry a molecule targeted to CFTR intron 9, allowing for trans-splicing at this locus and replacing the mutated region with the correct one either in vitro in human CF polarized airway epithelia or in vivo in human CF bronchial xenografts [30].

Design of antisense vectors

For the approaches discussed above to be effective, the antisense RNA needs to be delivered in relatively high concentrations. Thus the vectors should include a strong and efficient promoter, which is persistently active either ubiquitously or in a desired tissue. Furthermore, the gene cassette should express RNA molecules that are devoid of any inhibitory secondary structure, colocalize in the same subcellular compartment as the target sequence [31] and be resistant to intracellular nucleases. These criteria are met by snRNAs, making them appealing antisense vectors for modification of splicing.

SnRNAs are localized to the nucleus, are stable and are expressed from their own promoters at relatively high levels. Most importantly despite their structure they interact with their natural pre-mRNA targets by base pairing, i.e. via antisense interactions. Thus, replacement of the antisense sequences with those targeted to a desired pre-mRNA proved effective in the reports discussed above. Analysis of the data from the reviewed reports indicates that U1, U2 and U7 snRNAs are rather similar in their antisense activities. This is surprising since their natural targets and functions are substantially different.

U7 snRNA mediates 3′ end processing of histone pre-mRNA by base pairing the first 18 nucleotides at its 5′ end to the 3′ region of the target [32]. Therefore, U7 can be changed to a mediator of splicing by replacing the anti-histone pre-mRNA sequence with sequences antisense to the target splice sites. U7 snRNA is expressed at low levels ($\sim 10^4$ molecules/cell), but its nuclear accumulation can be increased by converting the U7 Sm site to the SmOpt sequence shared by more abundant snRNAs [33]. Moreover, this change eliminates U7's function in histone pre-mRNA processing, thus making it available for its new role of modulator of splicing [18]. U1 snRNA is driven by a strong promoter and, because it is transcribed from several genes, its levels reach 10^6 molecules/cell. U1 snRNA is involved in recognition of 5′ splice site sequences, suggesting that it could easily be converted to a modulator of pre-mRNA splicing by changing its binding sequence [32]. However, such a construct will be in direct competition with endogenous U1, which may prevail due to its high concentrations.

Consistent with this hypothesis, in correction of IVS2-705 thalassemic splicing, anti-5′ splice site U1 failed while that targeted to the cryptic 3′ splice was effective in restoring correct splicing of β-globin pre-mRNA [20]. Another possible antisense carrier, U2 snRNA, mediates the recognition of the intronic branch site and the entry of the catalytic U6 snRNA into the spliceosome. Again, this snRNA can be converted to a splicing modifier by replacing its binding sequence with an antisense sequence [28]. These three RNAs are transcribed by RNA polymerase II, while another possible antisense carrier, U6 snRNA, is transcribed by RNA polymerase III. In contrast to the other snRNAs U6 never leaves the nucleus and should be superior in modification of splicing, which proved not to be the case ([20] and M. Vacek, unpublished data).

The best way to deliver the antisense constructs to desired cells is by the use of viral vectors. These vectors are replication-defective viral particles that are able to enter the target cells and transfer their genetic material to the host genome. By replacing the genes required for viral replication with an expression cassette containing the antisense gene, the viruses become safe vectors. Several vectors have been employed to mediate antisense gene delivery, including retrovirus [28], lentivirus [21], adenovirus [30] and adeno-associated virus (AAV) [34]. Retroviral vectors can stably infect dividing cells by integrating into the host DNA; however, they are unable to infect nondividing cells. The human immunodeficiency virus (HIV)-based lentiviruses, in contrast, are able to infect both dividing and nondividing cells. The main disadvantage to these integrating vectors is the possibility of insertional mutagenesis; however, genetic modifications have been made to reduce this concern [35]. Adenoviruses can also infect non-dividing cells, but only confer transient gene expression, as the viral genome remains episomal [36]. Novel forms of these vectors do not induce immune response, which previously led to problems [37]. In contrast, no human pathology has been associated with AAV, which is able to infect certain nondividing cells, such as muscle, brain and liver. However, its use is limited by the small carrying capacity of its genome. AAV provides a very safe vector since it has not been associated with any human pathology [38].

Modification of splicing by antisense oligonucleotides

While antisense RNAs can be expressed for a long time if the constructs are incorporated into the genome [39], antisense oligonucleotides can persist in the cells for at most a few days [40]. Thus, antisense RNA treatments are a form of gene therapy while oligonucleotide treatment represents a pharmacological approach. Oligonucleotides have been used to shift pre-mRNA splicing of CFTR [41], IL-5R [42], c-myc [43], tau [44], SMN -2 [45], and bcl-x [16, 46, 47]. Here we focus on the

shifting of splicing of β-globin and dystrophin pre-mRNA.

β-globin

The mutations at positions 654, 705 and 745 of intron 2 of the β-globin gene (fig. 1) were targeted by 2′-*O*-methyl and morpholino oligonucleotides [11] and morpholino oligonucleotides. Recently, this treatment resulted in the ex vivo correction of β-globin pre-mRNA and up-regulation of hemoglobin in erythropoietic progenitor cells from patients with IVS2-654 and -745 thalassemia [23, 48]. Similar results [48] were also seen in a mouse model of IVS2-654 thalassemia [49]. Although doses of up to 45 μM were required to elicit the effect, sequence specificity and dose and time dependence were maintained. Furthermore, fluorescent-labeled oligomers showed that the compounds were efficiently entering the nucleus of the cells. Similar results were obtained with morpholino oligonucleotides targeted to the β^E aberrant splice site [50].

Dystrophin

As discussed above, antisense-induced skipping of the dystrophin exon containing the premature stop codon can restore the translational reading frame and generate functional protein, converting the severe DMD form to the less severe BMD form of the disease. A mouse model of DMD, carrying a missense mutation in exon 23 of dystrophin gene [51], has allowed the testing of antisense oligonucleotides in vivo. The 2′-*O*-methyl-oligoribonucleotide directed at the 5′ splice site of exon 23 proved effective when locally injected into mouse muscle in the presence of a cationic lipid [52]. Oligonucleotides targeted to an exon splicing enhancer-like sequence induced skipping of dystrophin exon 46 in myotube cells from two DMD patients who carried a deletion of exon 45, which disrupted dystrophin reading frame. Proper translation was restored when the additional exon 46 was also skipped. It was found that a 15% level of exon skipping was sufficient to restore normal amounts of properly localized dystrophin in at least 75% of myotubes [53]. The same group also investigated oligonucleotides targeted to mutations in other forms of the disease [54]. Recently, experiments have been undertaken to optimize the target sequence best suited to restore the reading frame of DMD pre-mRNA [55].

Chemically modified synthetic oligonucleotides

Several requirements must be met for modified oligonucleotides to be effective in shifting splicing: (i) the oligonucleotides must not activate RNase H-mediated destruction of the target pre-mRNA, (ii) they must be able to reach its site of action within the cell [11] and (iii) they must have high enough affinity to the target sequence to effectively compete for binding with splicing factors (reviewed in [11]). Over the past decade, a plethora of such compounds have emerged [56].

2′ Carbohydrate modifications

The addition of residues at the 2′ position of deoxyribose in the oligonucleotide prevents recognition of the oligonucleotide-RNA duplex by RNase H and destruction of the target RNA [57]. 2′-*O*-Me-oligonucleotides were the first to be used to shift splicing [22, 58]. More recently, a 2′-*O*-methoxyethyl (2′-*O*-MOE) phosphorothioate derivative [59, 60] was developed that has higher nuclease stability [61] and improved antisense activity. These compounds also show effects in vivo [62, 63]. Other 2′-modified compounds include the zwitterionic 2′-*O*-aminopropyl oligomer, which harbors a positive charge at the 2′ position in addition to the negative charge on the backbone [64, 65] or locked or bridged oligomers in which a methylene group bridges the 2′-*O* and 4′ positions of the ribose ring [66, 67]. This bridge locks the ribose ring in an N-type conformation, which is more favorable for binding RNA, imparts higher stability, RNase H inactivity and an increase in T_m of approximately +4–5°C/base. Locked oligomers have been used as antisense compounds for shifting splicing [68] or as triplex forming strand invaders [69].

Backbone modifications

Many backbone modifications exist, and most share certain characteristics, such as resistance to cellular and extracellular nucleases, the resistance of RNA:oligomer duplexes to degradation by RNase H and in most cases increased affinity for target sequences. Such oligomers include morpholino [70, 71], peptide nucleic acid (PNA) [72, 73], methylphosphonate (74) and phosphoramidate (NP) oligomers [75, 76]. These oligomers all represent improvements in chemistry and should be useful for shifting splicing.

The EGFP-654 antisense splicing assay

Most antisense assays involve measuring the downregulation of a given mRNA or protein, an approach that is affected by high and possibly variable background signal. Furthermore, nonspecific effects such as toxicity or protein binding can easily be mistaken as antisense effects in these cases. A better assay should be based on a positive signal with low background. The assay method should al-

low antisense testing with high throughput and sensitivity to and yield quantifiable results. Since the nucleus appears to be the major site of action for antisense oligonucleotides [11], the assay should be able to reflect the ability of a given oligonucleotide to accumulate and act in the nucleus. To this end, this lab has developed an assay based on splicing of the IVS2-654 mutant β-globin intron as a positive reporter system for antisense activity [77]. The IVS2-654 intron was inserted into the coding sequence of the enhanced green fluorescence protein (EGFP) gene and prevented EGFP expression due to aberrant splicing that mimics the splicing pathway of IVS2-654 thalassemia. Stable HeLa cell lines that express this construct do not produce EGFP unless splicing is corrected by antisense oligonucleotides. Generation of EGFP after antisense treatment is easily detectable and quantifiable by several methods. This assay provided convincing evidence that the neutral morpholino and PNA oligomers, the latter conjugated to four lysines, outperformed negatively charged 2′-modified phosphorothioate oligomers in free uptake experiments [77].

Recently the above functional assay was recapitulated in vivo in a transgenic mouse model. This model was used to investigate the antisense activity of chemically modified 2′-*O*-MOE phosphorothioates, morpholinos and PNAs containing four lysines at the 3′ end (PNA-4K). It was found that the PNA-4K exhibited the highest antisense activity after systemic delivery, upregulating EGFP in several tissues, including the liver, kidney and heart. PNA oligomers with only one lysine (PNA-1K) were completely inactive, suggesting that the 4-lysine tail is necessary for the antisense activity of PNA oligomers in vivo. The 2′-*O*-MOE oligomers also exhibited antisense activity in the above tissues as well as in the small intestine. The latter effect appears to be related to the intraperitoneal injection of the oligomers. In contrast, little efficacy was seen with comparable concentrations of morpholino oligonucleotides. The sequence-specific ability of PNA-4 and the 2′-*O*-MOE oligomers to upregulate EGFP strongly support that true antisense activity can be obtained by systemically delivered modified oligonucleotides, thus verifying them as potential macromolecular therapeutics. It is also notable that 2′-*O*-MOE were ineffective in cell culture but active in vivo, while the opposite was true for morpholino oligomers. Clearly, further investigations of oligonucleotide uptake mechanisms and the effects of pharmacodynamics on antisense activity are sorely needed.

Analysis of alternative and aberrant splicing with antisense oligonucleotides

Correction of aberrant splicing of IVS2-654 and IVS2-705 pre-mRNAs was observed after treatment not only with oligonucleotides targeted to the aberrant splice sites but also with the one complementary to a region centered around the nucleotide 623 of β-globin intron 2 (i.e. 31 and 82 nucleotides upstream from the aberrant 5′ splice site, respectively) [F. Gemignani, unpublished]. Mutagenesis experiments showed that a four-nucleotide insertion within this region corrected splicing of EGFP-654 [79]. These results suggest that the oligonucleotide or the mutation likely disrupted activity of a splicing enhancer sequence [80–82], thus simultaneously inhibiting aberrant and enhancing correct splicing.

Antisense oligonucleotides were also used to analyze the accessibility of the splice sites in IVS2-654, -705 or -745 pre-mRNAs. Differences in accessibility were demonstrated by treating the cells with a 17-mer antisense oligonucleotide (ON-3′cr) targeted to the common 3′, cryptic splice site activated by the mutations [83]. Although the same site was targeted in all three cell lines, the oligonucleotide exhibited dramatic differences in its EC_{50} (50% efficiency of correction) values for splicing correction in the three contexts. IVS2–654 pre-mRNA splicing was nominally corrected, with the EC_{50} of ON-3′cr at 1500 nM, while for the -705 and -745 pre-mRNAs the EC_{50} values were 30 nM and 2 nM, respectively. Changing the IVS2-654 and -705 splice sites to consensus 5′ splice sites (IVS2-654con, and -705con) led to additional decreases in accessibility of the 3′ splice site to ON-3′cr. IVS2-654con pre-mRNA did not respond to ON-3′cr at any concentration, while IVS2-705con responsiveness was reduced four-fold. The data suggest that the differences in effective concentration must have been due to differences in the ability of the same oligonucleotide to access that cryptic 3′ splice site. These data also support the exon definition model, which postulates interactions between the 3′ and 5′ splice sites to define an exon [84].

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ANTICANCER RESEARCH 23: 2007-2014 (2003)

Review

CaSm Antisense Gene Therapy: A Novel Approach for the Treatment of Pancreatic Cancer

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Abstract. *Pancreatic adenocarcinoma is a major clinical problem with few effective treatment options. In the United States 29,000 cases are diagnosed annually with an associated mortality rate greater than 90%. Given this dismal prognosis, a better understanding of the molecular controls that govern pancreatic cancer is clearly needed in order to develop more effective therapies. As such, our group has been actively investigating the identification and potential application of novel gene targets for this disease. We have recently identified the cancer-associated Sm-like (CaSm) oncogene, shown that it is overexpressed in 87% of human pancreatic cancer samples, and clearly demonstrated that it functions as a classic oncogene. We have also been able to show that an adenovirus expressing antisense RNA to the CaSm gene (Ad-αCaSm) is able to reduce endogenous CaSm mRNA expression and decrease anchorage-independent growth. A single intratumor injection of Ad-αCaSm extended survival in an in vivo SCID mouse model of human pancreatic cancer. To gain insight into the mechanism of Ad-αCaSm's anti-tumor effect, cell cycle studies were performed. Ad-αCaSm treatment of pancreatic cancer cells resulted in a cytostatic block with decreased G_1-phase and increased DNA content in vitro. Importantly, the combination of Ad-αCaSm with gemcitabine (an S-phase active chemotherapy) significantly extended survival time beyond either therapy alone. These studies have defined the CaSm oncogene as a novel gene target for therapy and have begun to define its potential role in the pathogenesis of pancreatic cancer.*

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Pancreatic cancer is the fourth leading cause of death from malignancy in the United States and represents a significant medical problem throughout the world. The disease often presents at an advanced stage, is resistant to all forms of therapy, and as a result has one of the highest mortality rates of any cancer(1). In light of this exceedingly poor prognosis, an effort has emerged to understand the molecular biology of this disease in order to develop more effective therapies. Examination of resected pancreatic cancer samples frequently reveals hyperplastic ductules with varying degrees of dysplasia adjacent to the tumor. The recently adopted pancreatic intraepithelial neoplasia (PanIN) pathological grading system stratifies these dysplastic lesions into low, intermediate and high grades (PanIN-1, PanIN-2, and PanIN-3, respectively). PanIN-1 lesions have low malignant potential and may never convert to overt carcinoma. PanIN-2 and PanIN-3 lesions, however, display greater cytological atypia, more architectural abnormalities, and frequently evolve to invasive cancer (http:www.path.jhu.edu/pancrease_painin). Numerous studies in the last decade have identified gene alterations involved in the various stages of this molecular progression (2-4), and these gene mutations have been extensively studied as methods for early detection and as targets for gene therapy (5-7). Promising initial results have been reported for k-ras, p53 and other gene therapies but, to date, no genetic therapy has proven clinically beneficial for the treatment of pancreatic cancer (8-10). Further studies are therefore needed to provide a better understanding of the molecular controls that govern pancreatic cancer in order to develop more effective therapies. Our group has been actively investigating a novel gene therapy approach based on the cancer-associated Sm-like (CaSm) oncogene.

The CaSm oncogene

In order to identify novel gene alteration in pancreatic cancer development, we began a project using subtractive hybridization to identify differentially expressed genes between the human pancreatic cancer cell line Capan-1 and

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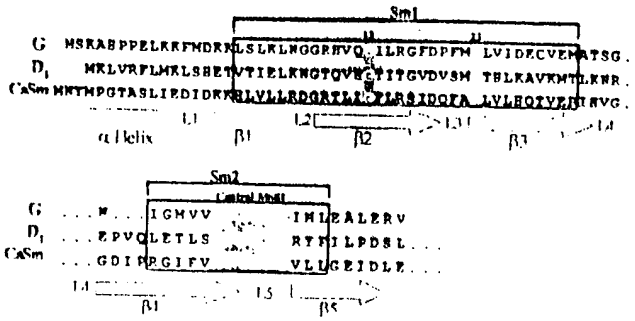

Figure 1. *Predicted structural domains of CaSm. The tertiary structure of several core Sm proteins has been resolved by X-ray crystallography. Based on these data, it is predicted that all proteins containing the Sm motifs will fold in a similar manner. The above diagram aligns the Sm motif regions of CaSm and the core Sm protein D_1 and Sm G. The positions of the predicted 5 β sheets are indicated with arrows. CaSm shares several conserved amino acids within the Sm motifs, which further indicates that CaSm will form a similar tertiary structure. Sm1 contains two invariant amino acids in all Sm containing proteins at positions 13 and 23 (highlighted in gray). Sm2 contains a central motif IRGXNI (highlighted in gray). CaSm also contains these invariant amino acids and most of the central motif in Sm2.*

the diploid, transformed but non-neoplastic pancreatic epithelial cell line HS680.PAN. The CaSm oncogene displayed a substantially higher signal in the neoplastic Capan-1 cell line compared to the normal pancreatic cells and a full-length cDNA clone was identified (11). Although the level of CaSm expression was variable between samples, fifteen of sixteen human pancreatic adenocarcinomas showed significant overexpression of a 1.2-kb CaSm mRNA in tumors relative to normal controls. This represents overexpression in 87.5% of samples (11) and is a very high frequency of oncogene overexpression in pancreatic adenocarcinoma. The frequency of CaSm involvement rivals that of k-ras mutation and is significantly more frequent than p53 mutation. Moreover, several human pancreatic cancer cell lines including AsPC-1, BXPC-3, Capan-1, Capan-2, COLO357, HPAC, MiaPaCa-2 and Panc-1 all overexpress CaSm. The CaSm mRNA was overexpressed in some samples of pancreatitis suggesting that the gene alteration may be an early event in tumor formation.

Furthermore, CaSm expression is not limited to pancreatic tumors. Cancer derived cell lines from bladder, kidney, liver, lung, ovary and rectum all overexpress CaSm mRNA (11). Although CaSm mRNA was also expressed to some degree in a variety of normal tissues, these levels of expression were markedly lower than in the corresponding tumors. Thus the oncogene is found elevated in a variety of cancer types and a low level of expression is observed in various normal tissues. This indicates that CaSm may have a normal function in a variety of cell types and that abrogation of this normal function may be a significant contributor to the pathogenesis of a variety of cancers.

Gene structure

The full-length genomic structure of CaSm has been determined in human and murine cells (Fraser and Watson unpublished data). CaSm is located on the short arm of chromosome 8 (8p11.2) between the BCL2-associated athanogene 4 (BAG4/SODD) and the steroidogenic acute regulatory protein (STAR) (www.ncbi.nlm.nih.gov Locus ID# 27257). The gene consists of 4 exons spread over a 14.5kb region (Fraser and Watson, unpublished data). The promoter region for CaSm has yet to be functionally characterized.

The CaSm gene encodes a mRNA transcript that is 1.2kbp in length with a polyadenylation signal at base pair 878-883. The translational start site is located at nucleotides 165-168. The largest open reading frame predicts a 133 amino acid polypeptide with a molecular weight of 15,179 and an isoelectric point of 4.97. CaSm was named "Sm-like oncogene" for the presence of a Sm motif in the sequence. An Sm motif is a conserved region of sequence homology that is thought to encode a region that functions in protein/protein interaction. The classical Sm motif is approximately 100bp in length and contains two Sm domains. Sm domain 1 is normally 32 amino acids long and contains a universally conserved glycine at position 13 and asparagine at position 23. There is a 10-30bp nonconserved linker region between the two Sm domains. Sm domain 2 is classically only 14 amino acids long and although highly conserved does not contain any invariant positions (12).

The CaSm sequence contains an Sm motif with two Sm domains at the expected positions (Figure 1). The CaSm Sm-1 domain is 32 amino acids long and overall, 12 of the 15 defined positions in the consensus of Sm domain 1 are conserved in CaSm including the 100% conserved glycine and asparagine residues. CaSm contains an 11 amino acid linker between its two Sm domains. Moreover, 10 out of 11 defined positions in Sm domain 2 are conserved in CaSm.

The Lsm family of proteins

Computerized BESTFIT sequence analysis of CaSm reveals a 32% identical and 60% similar homology with the human Sm G protein. This similarity led to the name "Sm-like oncogene" and may provide some information regarding the structure of CaSm(11). Sm G is part of a larger family of Sm proteins that form the spliceosome, an intricate complex of proteins that function in mRNA splicing. Recently the X-ray crystal structure of two Sm protein complexes made up of Sm D3/B

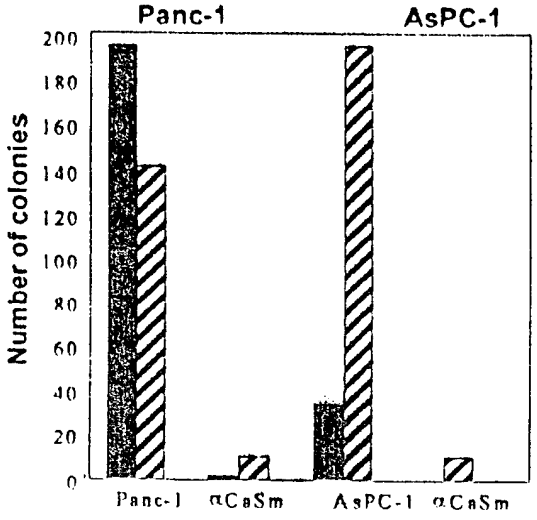

Figure 2. *CaSm antisense reduces the anchorage-independent growth of human pancreatic cancer cells. AsPC-1 and Panc-1 cells were stably transfected with CaSm antisense or a control plasmid and plated in a 1% agar solution. Cells were observed for the number and size of anchorage independent colonies 21 days after infection. The numbers of large colonies (>280um ▨) and small colonies (140-280μm ■) are shown. Results indicate a clear decrease in both large and small colonies after treatment with CaSm antisense.*

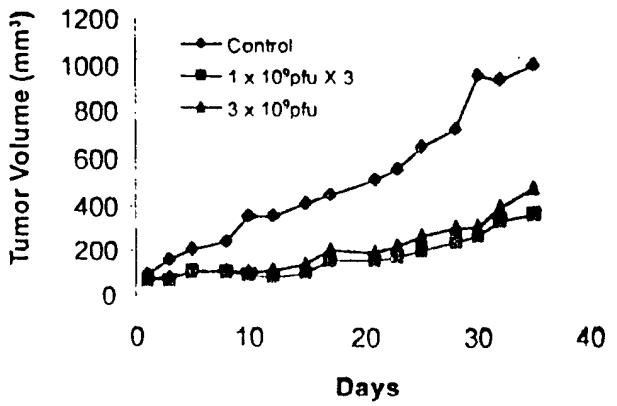

Figure 3. *Ad-αCaSm reduces subcutaneous tumor volume in SCID-Bg mice. Animals bearing subcutaneous AsPC-1 pancreatic tumors were injected on Day 0 with 100μl of saline containing Ad-LacZ (3 x 10^9pfu) or Ad-αCaSm (3 X 10^9pfu or 1 X 10^9 pfu given weekly for three weeks). Animals were monitored over time to determine the effect of this gene therapy on tumor volume. Treatment with Ad-αCaSm showed a clear decrease in tumor growth (n=10). A single large dose showed equivalent efficacy to three weekly doses.*

and Sm D1 D2 have been described(13). This structure demonstrates that Sm proteins are composed of a single helix followed by 5 anti-parallel β-pleated sheets (3 in Sm domain 1 and 2 in Sm domain 2). From this structure of the crystallized heterodimer, the β4 sheet of one protein is predicted to interact with the β5 sheet of a second protein(14). A subsequent report has expanded our knowledge of Sm protein structure and shown that the seven Sm proteins bind to one another to form a "barrel-shaped" scaffolding that is the basis of the snRNP(13). The homology between Sm G and CaSm is greatest in the Sm motifs and it seems likely that the oncogene may also form part of a "barrel-type" structure along with other Sm or Sm-like (Lsm) proteins.

While the Sm family of proteins functions in mRNA splicing, a similar family of Sm-like (Lsm) proteins plays a role in RNA decapping and degradation. Messenger RNA is normally produced in the nuclei and quickly modified by guanyl transferase, which adds a 7-methyl guanosine cap to the 5' end. Most mRNA sequences also contain an AAUAAA sequence near the 3' end. Poly-A polymerase binds to this sequence and adds a string of 50-200 adenosine residues to the end of the transcript forming a poly-A tail. The 5'-methyl guanine cap and the poly-A tail play important roles in stabilizing mRNA. Poly-A binding protein attaches to the poly-A tail of the nascent mRNA and stabilizes the message. Without a tail, the average mRNA half-life varies from less than 30 minutes to several hours. With a poly-A tail, mRNA half-lives range from several hours to even days. The 5'-methyl cap on an mRNA species also stabilizes the transcript by preventing degradation. Non-capped messages are rapidly degraded while capped mRNAs are far more stable.

The process of mRNA degradation occurs through a complex sequence of events that have been well-studied in yeast (15). The first step of the degradation process involves cleavage of the poly-A tail. In the absence of the tail, the poly-A binding protein disassociates from the mRNA transcript. Studies then indicate the Pat1 protein binds to the deadenylated mRNA and recruits a complex of Sm-like proteins(16). The Sm-like proteins (Lsm-1,2,3,4,5,6 and Lsm-7) are thought to form a seven-membered ring analogous to the "Sm-barrel" that functions in the splicesome. The Lsm-barrel allows the decapping proteins (Dcp-1 and Dcp-2) to bind to the deadenylated mRNA transcript and cleave the 7-methyl cap (17-21). The decapped, deadenylated RNA is then rapidly degraded by the exonuclease Xrn-1(22).

Recent studies have shown that CaSm is probably a member of the Sm-like family of proteins and in fact, CaSm

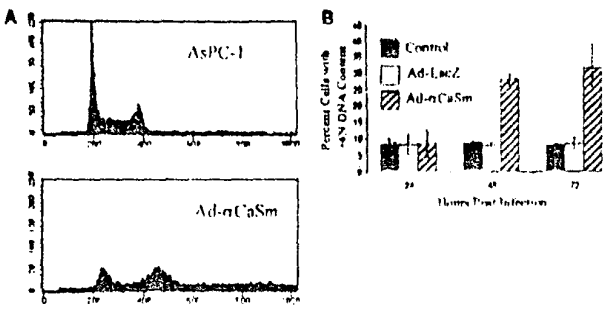

Figure 4. *Ad-αCaSm increases DNA content in pancreatic cancer cells. AsPC-1 cells display a striking change in the cell cycle after infection with Ad-αCaSm (Panel A). Infected AsPC-1 contain a significant increases in >4N DNA content relative to untreated cells or Ad-LacZ-infected cells (Panel B). (Modified from Kelley JR et al. Surgery 130: 280-288, 2001).*

has been designated human-like-Sm protein 1 (hLsm-1). This suggests that the oncogene may function to control mRNA stability in cancer. The yeast Lsm-1 protein (37% identical, 67% similar at the amino acid level to CaSm/hLsm-1) has also been described as Sph8 or suppressor of poly-A binding protein mutation-8 (22). As noted above, the poly-A binding protein (PABP) stabilizes mRNA and enhances transcription at the ribosome. Mutation in PABP leads to a dramatic decrease in translation that can be lethal to yeast. In this study, Sph-8 was discovered in strain of yeast that survived mutation in the poly-A binding protein. Sph8 mutants did not contain an increase in ribosome number and did not demonstrate an increased efficiency of transcription or translation. The cells were able to survive the PABP defect by increasing the stability of mRNA. Immunoprecipitation with anti-7-methyl cap antibody and Northern blot analysis indicate that Sph-8 cells accumulate deadenylated but capped mRNA transcripts. This strongly suggests that yeast Lsm-1 functions as a cofactor in the decapping process.

CaSm functions as a classical oncogene

The above studies suggest that CaSm functions in mRNA decapping and stability but further research is needed to confirm this hypothesis. The gene's role in pancreatic cancer, however, is better understood as it appears to function as a classical oncogene. CaSm transfected NIH3T3 cells form foci when plated *in vitro* (80 foci in CaSm transfectants *versus* 9 foci in untreated controls) and SCID mice injected with CaSm transfected NIH3T3 cells develop subcutaneous tumors (3/5 animals *versus* 0/10 NIH3T3 control animals. Fraser and Watson unpublished data). More importantly, CaSm is not only expressed at a very high frequency (87% of human pancreatic adenocarcinoma samples) but is required to maintain the transformed phenotype (Figure 2). When human pancreatic cancer cell lines are transfected with a plasmid that expresses antisense RNA for CaSm, the transfectants display a striking decrease in anchorage-independent growth (11). Anchorage-independent growth in soft agar is currently the best *in vitro* correlate of *in vivo* tumorigenicity. The ability of CaSm antisense to reduce this anchorage-independent growth indicates that the gene is necessary to maintain the neoplastic state and suggests that CaSm may be useful as a gene therapy.

CaSm-based gene therapy

To test the utility of CaSm as a novel target for gene therapy, an adenoviral vector was engineered to expresses antisense RNA to CaSm (Ad-αCaSm). Northern blot analysis of human pancreatic cancer cells infected with Ad-αCaSm indicates a substantial decrease in endogenous CaSm mRNA levels after infection (23). And more importantly, Ad-αCaSm-infected cells display a significant reduction of *in vitro* proliferation compared to controls. The effect of Ad-αCaSm was examined on a panel of human pancreatic cancer cell lines including: AsPC-1, BXPC-3, Capan-1, MiaPaCa-2 and Panc-1. The proliferation of all of the cell lines is decreased following infection with Ad-αCaSm and each cell line shows a dose response (23). Reduced CaSm expression also decreases anchorage-independent growth of the panel of cell lines when plated in soft agar (23).

The effect of Ad-αCaSm on an *in vivo* model of pancreatic cancer was also examined (Figure 3). To establish this model system, AsPC-1 cells were injected subcutaneously onto the flanks of the female SCID-Bg mice. After palpable tumors developed, these animals were then treated with a single intratumor injection of saline, Ad-LacZ or Ad-αCaSm. Ad-αCaSm has a dramatic effect on tumor volume reducing tumor growth by 40%, while treatment with the Ad-LacZ control virus did not substantially alter tumor size(23).

Animals were also monitored to determine the effect of CaSm antisense on survival. Treatment with Ad-αCaSm significantly prolongs survival in this model of pancreatic cancer. Mock-infected animals all died by 35 days post treatment. Animals treated with the Ad-LacZ control virus survive for 40 days post treatment. However, treatment with Ad-αCaSm prolongs the median survival to 60 days with some animals surviving for 100 days (23). In all cases, treatment with Ad-αCaSm is well tolerated by the animals. No mice showed signs of weight loss, decreased activity, or other signs of toxicity. There was a frequent hyperemia at

the site of injection in the tumor in both control and antisense-treated animals and occasional ulceration at the injection site was noticed in both groups. Four mice from control, Ad-LacZ and Ad-αCaSm treatment groups were sacrificed 30 days after injection and examined by histology for signs of pathological change. The livers, spleens, kidneys, pancreas, and tumors were removed, fixed in formalin, embedded in paraffin and examined by hematoxylin/eosin staining. No differences were seen between control and Ad-αCaSm-treated animals in this initial study of toxicity (Kelley *et al.*, unpublished results).

The mechanism of Ad-αCaSm's anti-tumor effect

To better understand CaSm-based gene therapy a series of experiments were designed to examine underlying mechanisms involved in the anti-tumor effect. These experiments began by determining if Ad-αCaSm induced apoptosis in treated cells. Agarose gel electrophoresis, TUNEL assay and activated Caspase-3 assays all failed to detect a significant degree of apoptosis in any of the treated cell lines after infection(24). Treated cell lines were then stained with propidium iodide to determine if Ad-αCaSm induced a cytostatic effect. Results indicate a dramatic alteration in the proportion of cells in the different phases of the cell cycle. At 24 hours, CaSm antisense treatment gave a significant decrease in the number of G_1 with a corresponding increase in the proportion of S-phase cells. Forty-eight hours after infection, the G_1 population remains decreased with a corresponding increase now seen in G_2/M cells.

Interestingly, an increase in the percentage of cells with nuclei containing greater than the normal 4N content of DNA was also observed (Figure 4). At 24 hours, only 8% of control or Ad-αCaSm-infected cells display nuclei with greater than 4N DNA content. Forty-eight hours after CaSm down-regulation, this number increases to 28% (8 and 7% for untreated and Ad-LacZ controls, respectively). Seventy-two hours post infection the greater than 4N population is still present with control and Ad-LacZ-treated cells displaying 7 and 8% greater than 4N cells while Ad-αCaSm treatment yields 31%.

These results demonstrate that the predominant mechanism of Ad-αCaSm's anti-tumor effect is a cytostatic inhibition of the cell cycle. This finding of a cytostatic block with an increase in DNA content gives insight into the function of CaSm within pancreatic cancer cells and indicates an unusual anti-tumor response. There is a modest if any induction of apoptosis and the cells are not arrested in one of the classical cell cycle checkpoints. Instead, CaSm antisense-treated cells appear to re-replicate their DNA in a phenotype similar to endoreduplication. To further examine this cell cycle effect, Ad-CaSm-infected cell lines were

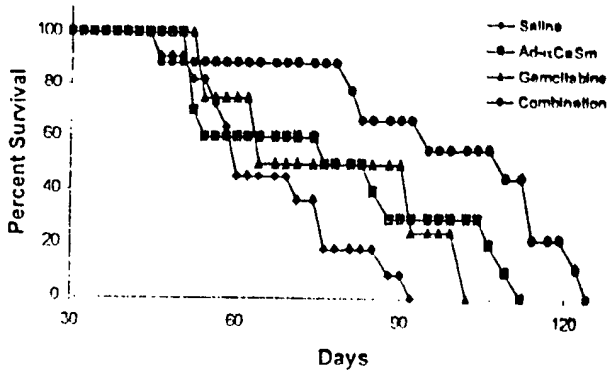

Figure 5. *Combination gemcitabine/Ad-αCaSm chemo-gene therapy significantly extends survival in a subcutaneous model of pancreatic cancer. Animals bearing subcutaneous AsPC-1 tumors were treated on Day 0 with a single intratumor injection of 100μl of saline containing Ad-LacZ or Ad-αCaSm. Animals also received 100μl intraperitoneal injections of saline or gemcitabine (40mg/kg) on Day 0, 3, 6, and 9. Animals were monitored over time to determine the effect of combination treatment on median survival. The combination chemo-gene therapy was clearly beneficial and extended median survival from 61 to 96 days. (Modified from Kelley JR et al. Surgery 130: 280-288, 2001).*

stained with DAPI and chromatin structure was examined by fluorescent microscopy. Results indicate that the chromatin of CaSm antisense-treated cells is condensed into chromosomes in the infected cells. This condensation is characteristic of prophase and shows that infected cells at least enter the first stage of mitosis. However, careful analysis of DAPI-stained cells demonstrates a striking lack of metaphase cells. In ten high-powered fields, control and Ad-LacZ-infected cells contain 17 and 19 metaphase cells, respectively. In sharp contrast, Ad-αCaSm-treated cells contain only 2 metaphase cells in 10 fields.

This data indicates that following a reduction in CaSm, cells enter the first mitotic stage of prophase but do not complete alignment in metaphase. This is an incomplete mitosis and argues that the endomitotic sequence is responsible for the increased DNA content after infection with Ad-αCaSm. In human megakaryocytes the endomitotic block occurs in an incomplete anaphase that seems to result from altered mitotic spindle dynamics. This appears to be a difference from the CaSm antisense effect. However, further experimentation is needed to more fully characterize the phase of the cell cycle where the CaSm antisense effect is predominant. We have not yet examined the spindle following infection with Ad-αCaSm. CaSm antisense may result in altered spindle dynamics that preclude metaphase alignment thus linking the two effects to a common site of defect. In addition, there are several markers of prophase,

prometaphase, metaphase and anaphase that could be used in future experiments to confirm a prophase block and study the other stages of mitosis. Experiments are currently underway to answer these questions.

Multi-modality therapy

Despite an incomplete understanding of the mechanism of Ad-αCaSm's anti-tumor effect, it is clear that decreased CaSm expression reduces pancreatic cancer cell growth in a cytostatic manner. This unique anti-tumor effect results from a decrease in G_1 phase and an increase in S-phase and DNA that immediately suggests a method of combination multi-modality therapy. An increase in the relative proportion of S-phase active cells with an accumulation of DNA suggests that a CaSm-based gene therapy may combine favorably with a cytotoxic chemotherapy that is active during synthesis. To test this hypothesis, CaSm antisense gene therapy was combined with gemcitabine chemotherapy and examined for effect on pancreatic cancer cell growth. The combination of Ad-αCaSm with gemcitabine results in a substantial decrease of *in vitro* proliferation in the AsPC-1 cell line *versus* single agent therapy (24). More importantly, this combination therapy is markedly more effective in an *in vivo* tumor model of pancreatic cancer (Figure 5). Treatment with gemcitabine in combination with an Ad-LacZ control virus reduces tumor volume by 35%. Ad-αCaSm alone decreases tumor size by 36% but the combination therapy reduces tumor volume by more than 70%(24).

Moreover, the multi-modality therapy significantly prolongs survival compared to either single agent. Untreated control animals die within 80 days in this model system with a median survival time of 60 days. Treatment with gemcitabine prolongs survival to 78 days, whereas Ad-αCaSm as a single agent produces a median survival time of 79 days. However, that combination of Ad-αCaSm with gemcitabine results in a median survival time of 100 days with some animals surviving for more than 120 days(24).

Conclusion

Pancreatic adenocarcinoma remains a major medical problem with an extremely high mortality rate. Lack of an effective therapy has led to an increased interest in novel treatment modalities to improve the management of this dismal disease. Recent studies by our group suggest that the cancer-associated Sm-like (CaSm) oncogene serves as a novel target in the pathogenesis of pancreatic cancer and that CaSm-based gene therapy may have potential. CaSm is overexpressed in more than 80% of human pancreatic cancer samples and a decrease in CaSm expression results in a decrease in pancreatic cell growth both *in vitro* and *in vivo*. The mechanism of this anti-tumor effect appears to be a cytostatic inhibition of the cell cycle with a corresponding increase in DNA synthesis activity. This mechanism of action allows Ad-αCaSm gene therapy to combine favorably with gemcitabine chemotherapy resulting in an additive decrease in tumor growth and a significant survival advantage.

Thus CaSm represents a novel target with exciting potential as a new treatment approach for pancreatic cancer. Further work is necessary to more fully describe the normal function of CaSm and the mechanism of Ad-αCaSm's anti-tumor effect. Additional studies combining CaSm-based gene therapy with other chemotherapeutic agents are currently underway as are experiments to test the utility of CaSm as an early detection.

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Molecular Therapeutics of HBV

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Abstract: The hepatitis B virus (HBV) infection is a public health problem worldwide, particularly in East Asia. The current therapy of HBV infection is mostly based on chemical agents and cytokines that have been shown to provide limited efficacy and are also toxic to the human body.

Gene therapy is a new therapeutic strategy against HBV infection, involving the transmission of gene drugs into liver cells by specific delivery systems and methods. Although this new anti-HBV infection technique is under active investigation, various promising anti-HBV viral gene drugs have been developed for gene therapy, including antisense RNA and DNA, hammerhead ribozymes, dominant negative HBV core mutants, single chain antibody, co-nuclease fusion protein, and antigen. In order to optimize their antiviral effects and/or enhance anti-HBV immunity, various novel gene delivery systems have also been developed to (specifically) deliver such DNA constructs into liver cells; some of them are viral vectors, such as adenoviral vectors, retroviral vectors and poxviral vectors, and even hepatitis B viral for its hepatocellular specificity. Others are non-viral vectors, in which naked DNA and liposomes are frequently used for DNA vaccine or nucleotide analogs for inhibiting HBV DNA polymerase.

This review addresses various aspects of gene therapy for HBV infection, including gene drugs, delivery methods, animal model, and liver transplantation with combination therapy. It also discusses the problems that remain to be solved.

1. INTRODUCTION

Despite the availability of efficient vaccines for protecting previously unexposed individuals, hepatitis B virus (HBV) infection remains a major cause of morbidity and mortality worldwide. There is significant geographic variation in infection rates, but it is estimated that 350 million people worldwide have chronic HBV infection [Lee, 1997]. In Southeast Asia, Africa and China, more than 50% of the population is infected, and 8% to 15% have become chronically infected. Chronic HBV infection is the cause of up to 50% of cirrhosis cases and 70%-90% of hepatocellular carcinoma (HCC) in these regions [Lok, 1992; Fattovich, 1998]. Between 250,000 and 300,000 new HBV infections occur annually, and 4,000 to 5,000 persons die annually from cirrhosis or liver cancer in the United States. Neonatal HBV infection nearly always results in chronic HBV infection. Pre-existing immunosuppression also increases the risk of chronic infection. Because the prevalence of infection is largely determined by a feedback mechanism, and the carriers are the most influential, predictive quantity, reduction in carrier prevalence via therapeutic treatment can be a means of controlling the incidence of HBV quickly relative to the effect of immunization and behavior modification [Medley *et al.*, 2001].

Chronic hepatitis B is the result of a continuing attack on infected cells by the host immune system, which is not vigorous enough to eradicate all the infected hepatocytes [Chisari, 1995]. Therefore, two main non-exclusive strategies can be envisaged to eradicate viral infection: inhibition of viral replication and/or nonspecific inhibition of viral replication combined with enhancement of immune response [Zoulim, 1999]. Currently, the only therapy for chronic hepatitis B with a lasting beneficial effect is the systemic treatment with interferon (IFN-α having dual properties, in that it inhibits viral replication and also stimulates cell mediation immunity to HBV by mechanisms that have not been fully delineated. The sustained response is achieved in only one third of patients [Hoofnagle and Bisceglie, 1997]. Any patients with chronic hepatitis B and markers of active viral replication, elevated ALT level and chronic hepatitis on liver biopsy are potential candidates for IFN-α therapy. HBV genotype C, compared to genotype B, is associated with a higher frequency of core promoter mutation, and a lower response rate to interferon alpha therapy [Kao *et al.*, 2000]. In addition to its severe side-effects [Hoofnagle and Bisceglie, 1997; Main *et al.*, 1998], IFN-α exhibits a short half-life in blood after parenteral protein administration [Heremans *et al.*, 1980], which limits its performance, as it is unable to make sufficient or sustained deliveries of the protein into the liver. Recently, nucleoside analogs have provided a therapeutic alternative, leading to a rapid decrease in serum HBV DNA levels and to a histologic improvement in the treatment of liver disease [Lai *et al.*, 1998; Urban *et al.*, 2001]. Clinical data show that lamivudine is an effective treatment for a wide range of patients with chronic hepatitis B, whose advantages include lower cost, peroral administration and a high r patient tolerance than IFN [Yao, 2000], however, short-term treatment leads to a rapid relapse of the disease, and long-term treatment often results in the developing of drug toxicity [Lee, 1995] or the selection of resistant viral variants

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[Zoulim and Trepo, 1998]. These outcomes emphasize the need for novel therapeutic approaches [Hoofnagle and Bisceglie, 1997]. In fact, the more advances in our understanding of the mechanisms underlying virus life histories, the more opportunities for the rational design of molecules that could specifically block viral infection, replication and maturation. Moreover, a combination of therapies with different mechanisms of actions may be most effective and prevent the selection of resistant viral strains. Genetic therapy may be one such therapeutic approach, as many transgenes have been shown to inhibit HBV *in vitro* or *in vivo*.

2. MOLECULAR DRUGS

2.1 Antiviral Strategies

2.1.1 Anti-Sense Nucleotide

Besides the study of cellular and viral gene functions, active research on antisense nucleotides is also underway, for their potential to interfere with viral gene expression as antiviral agents. The processes of replication, transcription, and translation of HBV can be blocked by antisense molecules specifically binding to target genes to inhibit viral actions. Previous studies have shown that a number of antisense oligonucleotides against HBV mRNA are able to inhibit viral gene expression *in vitro*. Many of these effective molecules, whether chemically synthesized or endogenously expressed, targeted almost all the specific functional sequences of viral genes. In the cell culture system, antisense DNA directed to HBV polyadenylation signal [Nakazono *et al.*, 1996; Wu *et al.*, 1992], 5'-upstream sequences [Nakazono *et al.*, 1996] and X gene [Feng *et al.*, 1997], and antisense RNA complimentary to preS/S [Tung and Bowen, 1998; Ji *et al.*, 1997; Wu and Gerber 1997], preC/C [Ji *et al.*, 1997; Soni *et al.*, 1998], HBV X and HBV P [Wu *et al.*, 2001], are particularly effective. Without an identical model, it is not certain which target is resultantly more effective. Korba and Gerin examined the ability of 56 different single-stranded oligodeoxyribonucleotides (14-23 nucleotides in length), which target several HBV-specific functions, to inhibit HBV replication in the human hepatoblastoma cell line 2.2.15. The oligomers directed against the HBV encapsidation signal/structure (epsilon) showed the most effective antiviral efficacy against HBV [Korba and Gerin, 1995]. Dual-target antisense RNA, expressed by retroviral vector in HepG2.2.15 cells, also exhibited higher inhibitory effect than its single-target counterparts [Wu *et al.*, 2001]. *In vivo*, an antisense oligodeoxynucleotide directed against the 5'-region of the preS gene of DHBV inhibited viral replication and gene expression in Peking ducks [Offensperger *et al.*, 1998]. Furthermore, poly-DNP-RNA with antisense RNA targeted against the DHBV polymerase gene could completely inhibit duck viremia, and thus viral DNA disappeared [Xin *et al.*, 1998]. Another antisense oligomer, complementary to the cap site of the SP II promoter of HBV mRNA, also produced an effective inhibitory effect, after injected into athymic nude mice producing HBV markers [Yao *et al.*, 1996]. Putlitz and Wands compared sense and antisense RNAs on HBV replication, and found that both inhibited HBV replication, but only sense sequence inhibited HBsAg secretion [Putlitz *et al.*, 1999]. This demonstrated that both sense and antisense base strategies could be successfully used to inhibit viral replication [Ding *et al.*, 1998].

A new concept of antisense is covalently linked to Ribonuclease H to obtain a direct specific cleavage event. It has been tested in tube to cleave HBV mRNA with the sequence recognizing ability of oligonucleotide specific for the DR1 region linked with RNase H [Walton *et al.*, 2001].

2.1.2 Ribozyme

Ribozymes act as RNA-cleaving RNA molecules that can catalyze the cleavage and inactivation of other cellular and viral RNA molecules with a specific nucleotide sequence. Their success *in vitro* is unquestioned, and the use of ribozymes, especially small catalytic RNAs, in antiviral gene therapy is also being actively pursued [Welch et al, 1998; Wong-Staal *et al.*, 1998; Macpherson *et al.*, 1999; Amado *et al.*, 1999]. The hammerhead ribozyme and the hairpin ribozyme have been designed to inhibit HBV gene expression, and have been analyzed in a cell culture system. Presently, a chemically modified ribozyme, targeting HBV mRNA, is at the clinical development stage. The main challenge for the use of ribozyme is the search for accessible target sites on a substrate RNA. A combinatorial screening method has been used to identify catalytically active hairpin ribozymes that mediate intracellular antiviral effects on HBV [Zu *et al.*, 1999]. Several hammerhead ribozymes also have been used with varying success to inactivate HBV RNA. The targeted sequences include poly (A) signal sequence [Feng *et al.*, 2001], the tail region of the HBV core protein [Feng *et al.*, 2001], and dual sites in HBc RNA [Li *et al.*, 2000]. Three hairpin ribozymes have been designed to target the pgRNA and specific mRNAs encoding the HBsAg, the polymerase and the X protein, and cotransfected into HuH-7 HCC cell together with small amount of an HTD of HBV in transfected human hepatocellular carcinoma (HCC) cells. As a result, the virus particles-associated HBV levels were reduced up to 83% [Welch *et al.*, 1997]. HBx RNA is the most plausible target for the ribozyme to block the HBV replication because the sequence of the smallest X transcription is fully included in the 3' sequence of all HBV transcription. Using ribozyme-encoding vectors to transfect liver cells, two research groups found that hammerhead ribozyme targeting to HBx sequence cleaved the substrate in a catalytic manner [Passman *et al.*, 2000; Kin *et al.*, 1999], however, Weinberg et al suggested that an antisense mechanism without substrate cleavage might be the dominant intracellular effect [Weinberg *et al.*, 2000].

Previous unsuccessful attempts [von Weizsacker *et al.*, 1992; Beck *et al.*, 1995] at using hammerhead ribozymes for the intracellular inhibition of HBV revealed that activity-selected ribozymes are likely to be more effective than sequence-selected ones, with respect to intracellular inhibitory effects. Extra sequences endogenously coexpressed with ribozyme may have an effect on the folding of the RNAs, and thus affect ribozyme formation and its accessibility. A recent report showed that the insertion of trans-ribozyme between two cis-ribozyme sequences led to the removal of the extra sequences and the abolishment of any cis-inhibitory effect from the non-ribozyme sequence [Feng *et al.*, 2001]. This argues that the level of the ribozyme expression is correlated with its inhibition efficiency in

cultured cells, which was found in a previous study [Kin *et al.*, 1999]. And this outcome indicates that a more effective delivery system is required in anti-HBV gene therapy. According to a published work on HIV, Rev-binding RNAs efficiently block HIV-1 gene expression, whereas other antisense RNA and ribozymes have little or no effect when expressed in the same cassettes. This observation demonstrates that different promoters should be chosen when expression cassettes are constructed to express different antisense RNAs and ribozymes in order to transcribe them efficiently, stabilize them against rapid degradation, fold them correctly, and deliver them directly to appropriate part of a cell [Good *et al.*, 1997].

The efficiency of ribozyme action in the complex intracellular environment is difficult to predict. Development of therapeutic sequences is often guided by the empirical assessment of intracellular functional inhibition of a target [Weinberg *et al.*, 2000], and thus is not always reliable. Plasmids containing intact HBV sequences or a modification in which the preS2/S region was replaced by DNA encoding enhanced green fluorescent protein (EGFP) were used to test ribozyme action in transfected cells. The measurement of EGFP expression is convenient to assess ribozyme action *in situ* [Passman *et al.*, 2000].

2.1.3 Triplex Forming Oligodeoxynucleotide

Unlike an antisense oligonucleotide, a triplex forming oligodeoxynucleotide (TFO) acts directly on the gene by binding to duplex DNA in a stable, sequence-specific manner. This oligonucleotide-directed triplex DNA formation has been shown to inhibit transcription factor binding to purine-rich motifs, and the TFOs have been used in this way to block transcription of various genes *in vitro* and in intact cells [McGuffie *et al.*, 2000; Jendis *et al.*, 1998]. Gao *et al.* designed a TFO that can form triplex with SP1 sites in HBV core promoter. When transfected into HepG 2.2.15 cells by packing with liposome, the synthesized TFO can effectively inhibit replication of HBV [Gao *et al.*, 2001]. The blocking of viral replication at transcriptional level with TFOs is a very promising molecular approach, although results in HBV infection are rare.

2.1.4 Aptamer

Many aptamers are designed to block protein functions. Aptamer, a small nucleotide, can bind to its ligand (protein, ion, antibiotic, etc.) with high affinity and high specificity. Their binding is due to their 3-D conformation. There is no sequence complementation between an aptamer and its ligand. Recently eight peptide aptamers were isolated from a randomized expression library, which specifically bound to the HBV core protein under intracellular conditions. One of them inhibited HBV replication by blocking viral capsid formation. This provides a new basis for the development of therapeutic molecules with specific antiviral potential against HBV infection [Butz *et al.*, 2001]. Accumulated findings about interaction between cellular proteins and specific sequences of HBV gene may give some hints for application of this new approach. Since interaction between nuclear receptors and the nuclear receptor response elements (NRREs) present in the HBV genome may play critical roles in regulating its transcription and replication during HBV infection of hepatocyes [Yu *et al.*, 2001], an aptamer can be designed to express in nucleus to compete for binding to the nuclear reporter proteins, and may result in anti-HBV infection. Similarly, aptamers binding to YY1, a transcription factor, may prevent HBV genome integrating into the cellular DNA, according to the report that integrated hepatitis B virus DNA preserves the binding sequence of YY1 at the virus-cell junction [Nakanishi-Natsui *et al.*, 2000]. For HBV pregenomic (pg) RNA to be encapsidated, its 5' end is folded into a stem-loop structure, that is the encapsidation signal (epsilon), which is involved in the activation of polymerase [Kramvis and Kew, 1998]. This offers the possibility of inactivating HBV polymerase protein by an aptamer, probably a more attractive and effective approach.

2.1.5 Nucleoside Analog

Nucleoside analog replaces naturally occurring nucleosides such as adenosine, guanosine, cytidine, and thymidine and uridine, and causes DNA chain termination. Using the avian HBV model system, Urban *et al.* obtained new insights into the catalytic mechanism of HBV reverse transcriptases (RT). It was shown that pyrophosphate (PPi)-dependent RT activities were able to efficiently remove newly incorporated nucleotides and certain antiviral drugs even under low, cytoplasmic concentrations of PPi. These activities operating during viral replication could potentially undermine the efficacy of some drugs. Analysis of chain-terminated DNA revealed that the potent anti-HBV drug lamivudine (3TC) was difficult to remove by pyrophosphorolysis, in contrast to ineffective chain terminators such as ddC. Therefore, it was suggested that HBV-RT pyrophosphorolysis activity may be a novel determinant of antiviral drug efficacy, and could serve as a target for future antiviral drug therapy [Urban *et al.*, 2001].

Nucleoside analog drugs suppress the replication but do not eradicate the HBV. As a result, stopping the medication may lead to a relapse of HBV. Lamivudine therapy induces improvements in chronic hepatitis B in a high proportion of patients, but prolonged therapy is limited by the development of viral resistance [Zoulim and Trepo, 1998]. Clinical data showed that long-term therapy with lamivudine resulted in sustained improvements in virologic, biochemical, and histologic features of disease in most patients with HBeAg-negative chronic hepatitis B and in the subgroup of HBeAg-positive patients with high serum transaminase levels. A high rate of resistance limited efficacy, particularly in patients who remained HBeAg positive on therapy [Lau *et al.*, 2000]. The HBV-specific CTL response before and during lamivudine therapy was studied longitudinally in 6 HLA-A2-positive patients with HBeAg+ chronic hepatitis B. This study shows that lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B [Boni *et al.*, 2001]. With the use of lamivudine, induced antiviral immune responses and consequent viral elimination have been observed in chronic hepatitis B patients who received six monthly intradermal vaccinations with HBsAg or together with daily Interleukin-2 (IL-2) s.c. [Dahmen *et al.*, 2002]. These data demonstrate that a cure for chronic HBV infection may be achieved by treatment with lamivudine in combination with immune therapies.

The FDA approved Lamivudine, a nucleoside analog, in 1998 for the treatment of chronic HBV infection. Nucleoside

analogs of this type directly block HBV polymerase-reverse transcriptase and inhibit viral replication. Treatment with lamivudine at a dose of 100 mg given orally once daily results in a rapid decrease in the HBV DNA level and marked improvement in measures of liver injury [Mailliard and Gollan, 2003]. The major problem with lamivudine monotherapy has been the emergence of drug-resistant HBV polymerase (YMDD) mutants. As a result, long-term use of lamivudine in other settings remains controversial [Perrillo, 2002]. Newer nucleoside analogs are being extensively investigated by studies *in vivo* and *in vitro*. According to *in vitro* studies, the resistance of HBV DNA polymerase mutants M552I, M552V to lamivudine triphosphate with inhibition constants (Ki) increased compared with that of wild-type HBV DNA polymerase. Encouragingly, these mutants remained sensitive to adefovir diphosphate, with the inhibition constants increasing 1.3 times and 2.2 times [Xiong *et al.*, 1998]. In pre-clinical and phase 2 studies of patients with HbeAg-positive chronic hepatitis B, 48 weeks of 10 mg or 30 mg of adefovir dipivoxil per day resulted in histologic liver improvement, reduced serum HBV DNA and alanine aminotransferase levels, and increased rates of HbeAg sero-convension. The 10 mg dose has a favorable risk-benefit profile for long-term treatment. No adefovir associated resistance mutations were identified in the HBV DNA polymerase gene [Marcellin *et al.*, 2003]. Perrillo *et al.* [2000] also demonstrated that adefovir dipivoxil was effective against lamivudine-resistant hepatitis B virus (HBV). Five patients with chronic HBV infection developed resistance to lamivudine after 9 to 19 months of treatment, and were then treated with adefovir dipivoxil in a dose of 5 to 30 mg daily. Two to 4 log (10) reductions in HBV-DNA levels were observed in 4 cases, and the fifth patient becomes negative by quantitative polymerase chain reaction after retransplantation in conjunction with hepatitis B immunoglobulin (HBIg). Combination therapy with two or three nucleotide analogs will become one of the main treatments of chronic hepatitic B in future.

2.1.6 Peptide

Peptide may be used as a therapeutic antigen by interfering with the interaction between HBV particles and the cell surface or the viral replication and maturation. The studies on components associated with the internalization of HBV particles may prove to be very useful to protect cells from infection, and consequently block viral replication. The existence of a fusogenic sequence was predicted in the junction area of the PreS2- and S-domain of the hepatitis-B virus surface antigens. Evidence has been produced that the sequence 7-18 of the hepatitis B S domain [Berting *et al.*, 2000], and motif amino acids 41 and 52 of PreS2 [Oess and Hildt, 2000] mediated cell-permeability. They may initiate the first step of viral entry. Correspondingly, the domain of the cellular reporter, carboxypeptidase D [Tong 1999], was identified, which may play a role in the binding and presentation of proteins or peptide substrates [Aloy *et al.*, 2001]. These efforts have increased the chances for the design of a peptide to block viral infectivity. Recently, a myristoylated Pre-S peptide was used in DHBV model. Though lacking in the essential part of the carboxypeptidase D receptor binding site, the peptide binds hepatocytes and subsequently blocks DHBV infection [Urban and Gripon, 2002].

Peptide ligands that bind to the core antigen of hepatitis B virus (HBcAg) were selected from a random hexapeptide library displayed on filamentous phage. *In vitro*, one of them inhibited the interaction between HBcAg and the pre-S region of the L polypeptide, which is critical for virus morphogenesis. The result suggested that this peptide and the related small molecules might inhibit viral assembly [Dyson and Murray, 1995]. Further study confirmed that the interaction of L-HBsAg with core particles was critical for HBV assembly, and demonstrated in principle its disruption *in vivo* by small molecules [Bottcher *et al.*, 1998]. It was shown that two distinct segments of the hepatitis B virus surface antigen contribute synergistically to its association with the viral core particles [Tan *et al.*, 1999]. In binding assays *in vitro*, it was found that empty HBV core particles bound synthetic peptides corresponding to HBV envelope protein domains with the same affinity as did HBV DNA-containing core particles [Hourioux *et al.*, 2000]. Watts *et al.* studied the morphogenic properties of the peptide STLPETTVV, which could influence the HBV capsid protein assembly. It was suggested that linker peptides were attached to the capsid inner surface as hinged struts, forming a mobile array, an arrangement with implications for morphogenesis and the management of encapsidated nucleic acid [Watts *et al.*, 2002]. A suitable vector for the delivery of these peptides deserves to be found, and their antiviral efficacy should be evaluated *in vivo*.

2.1.7 Chimeric Core Protein

The restriction of HBV genome replication to the nucleocapsid makes this nucleoprotein particle an attractive target for intervention. Dominant negative (DN) core protein variants have been shown to interfere with nucleocapsid assembly. In animal model systems, transient expression of the DHBV molecular equivalent of the WHV and HBV DN constructs inhibited wild-type (WT) DHBV replication by 95% [Scaglioni *et al.*, 1996]. Von *et al.* [von *et al.*, 1996; 1999] fused DHBV Pol, DHBV S, lacZ and GFP, respectively, to the carboxyl terminus of the DHBV core protein to yield DN mutants that inhibit viral replication in the avian hepatoma cell line LMH. Core-Pol and core-S, but not core-lacZ or core-GFP, markedly interfered with RNA pregenome packing, while the DN core-GFP fusion protein formed mixed particles with WT core protein and interfered with reverse transcription of the viral pregenome. The result suggested that DN DHBV core proteins could target at least 2 steps within the viral life cycle, packaging of the viral pregenome and reverse transcription within mixed particles [von *et al.*, 1999]. More recent report showed that recruitment of core protein to the DHBV preassembly complex occurs in a cis-preferential manner. This mechanism may account for the leak of DN DHBV core protein mutants targeting reverse transcription [Von *et al.*, 2002].

A conceptually more powerful approach is capsid-targeted viral inactivation, which exploits a viral capsid protein or other virion-associated protein as a carrier to bring a degradative enzyme specifically into virus particles

[Natsoulis and Boeke 1991]. Beterams and Nassal [2001] found that C proximal fusion to the HBV capsid protein of the Ca^{2+}-dependent nuclease (SN) yields a chimeric protein. In HBV co-transfected human hepatoma cell, less than 1 coreSN protein per 10 WT capsid protein subunits reduced titers of enveloped DNA containing virions by more than 95%. Furthermore, no evidence was found that coreSN is cytotoxic. The calcium signaling, involved in stimulation of transcription and viral DNA replication [Bouchard *et al.*, 2001], initiates the antiviral action of the coreSN in the infected liver cell [Beterams and Nassal, 2001].

As intracellular immunogens, chimeric core proteins may induce cytotoxic T cells. It should be clarified whether their induction would soon abolish their antiviral efficacy or, by contrast, would further contribute to virus elimination by concomitantly inducing a response against WT core protein in the infected cells.

2.1.8 Single Chain Antibody

Several published reports have demonstrated that antibody genes can be expressed inside cells where the corresponding antibody fragments bind to their targets with high affinity and thus efficiently interfered with the function of cellular targets [Marasco *et al.*, 1993; Mhashilkar *et al.*, 1995, 1997; Cattaneo *et al.*, 1999]. A study using a cloned single chain Fv (sFv) fragment directed against HBsAg showed that this antibody fragment could reduce extracellular HBsAg levels by a mean of 85%. Confocal microscopy studies confirmed the intracellular expression and colocalization of the sFv and HBsAg [zu *et al.*, 1999]. A man-made antibody anti-HBc sFv could also inhibit viral replication intracellularly by forming sFv-HBc complex and interfering with the function of HBc [Yamamoto *et al.*, 1999]. Using the purified Pol protein to raise monoclonal antibodies (Mabs), Putlitz *et al.* generated six Mabs directly against HBV Pol protein, of which a Mab specific for the Pol terminal protein region appeared to inhibit Pol function in the *in vitro* priming assay. This represents an important first step towards the further exploration of the intracellular antibody strategy against HBV [zu *et al.*, 1999]. It remains to demonstrate their capacity for inhibiting viral replication *in vitro* and to find convenient ways for gene delivery *in vivo*.

2.1.9 Alpha-Glucosidase Inhibitors

One function of N-linked glycans is to assist in the folding of glycoproteins by mediating interactions of the lectin-like chaperone proteins calnexin and calreticulin with nascent glycoproteins. These interactions can be prevented with inhibitors of the alpha-glucosidases, such as N-butyl-deoxynojirimycin (NB-DNJ) and N-nonyl-DNJ (NN-DNJ), and caused some proteins to misfold and retain within the endoplasmic reticulum (ER). Evidence was given that M protein of HBV folded via a calnexin-dependant pathway [Werr and Prange, 1998]. The presence of NB-DNJ virions and the M protein were retained surprisingly [Mehta *et al.*, 1997], and proper intracellular routing of HBV glycoproteins was disrupted in cells where ER glucosidase was inhibited [Lu *et al.*, 1997]. In a woodchuck model of chronic HBV infection, the NN-DNJ-induced misfolding of HBV enveloped glycoproteins prevented the formation and secretion of infectious enveloped virus. This provided the first evidence that glucosidase inhibitors could be used *in vivo* and had anti-viral effects [Block *et al.*, 1998]. Further studies showed that NN-DNJ retained antiviral activity at concentrations that had no significant impact n ER glucosidase function. In addition, N-nonyl-deoxy-galactojirimycin (N-nonyl-DGJ), an alkyl derivative of galactose with no impact on glycoprocessing, retained anti-HBV activity. These results suggested that NN-DNJ possesses an antiviral activity attributable to a function other than an impact on glycoprocessing [Mehta *et al.*, 2001]. Therefore the mechanism of the alpha-glucosidase inhibitor action should be further elucidated to facilitate the application of these antiviral agents. They have already been demonstrated to have an antiviral efficacy against several viruses [van *et al.*, 1996; Zitzmann *et al.*, 1999; Wu *et al.*, 2002].

2.2 Immune Modulatory Strategies

2.2.1 Cytokines

Since systemic application of cytokines is associated with severe side effects, researches on targeted delivery or endogenetic expression through a gene therapy approach, have been prompted. Eto and Takahashi prepared an asialoglycoprotein (ASGP) receptor-directed interferon and compared its antiviral effects with that of conventional natural human IFNs. Their study demonstrated that directing IFN to ASGP receptor facilitated its signaling in the liver and augmented its antiviral effect [Eto *et al.*, 1999]. Man-made Anti-HBsAg interferon fusion proteins displaying both IFN activity and HBsAg have prompted an alternative way of making a targeting drug for hepatitis B [Tong *et al.*, 2001; Xia *et al.*, 2002]. Local production should provide IFN more efficient and better tolerant [Aurisicchio *et al.*, 2000]. Protzer *et al.* constructed a recombinant DHBV carrying the duck homolog of IFN-α, and superinfected DHBV-positive hepatocytes with rDHBV-IFN *in vivo*. DHBV-production decreased relatively to untreated controls, in a dose-dependent fashion, comparable to the maximal effect observed in the treatment with the IFN protein. No change in DHBV progeny production was seen on superinfection with rDHBV-GFP, indicating that the transduced IFN gene caused inhibition [Protzer *et al.*, 1999]. In an acute hepatitis model, the hepatic damage by mouse coronavirus MHV-3 infection was reduced by help-dependent adenovirus HD-IFN vector expressing mIFN-α 2. Challenged with ConA, the HD-IFN injected mice were protected, as HD-IFN exhibited a protective effect against liver injury even at doses that do not yield circulating mIFN-α 2 level [Aurisicchio *et al.*, 2000]. Recently, evidence has been produced that enhanced interferon-stimulated gene factor-3α (p48) expression increases IFN-α-induced suppression of HBV RNA significantly, in an experiment based on human hepatoma cells [Rang and Will, 2001]. This indicates that in order to optimize the IFN-α effect, interferon-stimulated response like element (ISRE) and the interferon-stimulated gene factor (ISGF) may be taken into account. Furthermore, ISGF was found to bind to ISRE-like sequence identified in the linker regions located between the heptameric tet operator sequence, resulting in IFN-α-mediated tet promoter stimulation activity. The data imply that the tet promoter-based expression system can be rendered non-responsive to IFN-α by mutagenesis of the ISREs, and this may be

essential when considering gene therapy *in vivo* [Rang and Will, 2000].

Other immunomodulatory cytokines, such as IL-12, IFN-α, or tumor necrosis factor-α, have potently suppressed HBV replication in an HBV transgenic mouse model [Guidotti *et al.*, 1996; Cavanaugh *et al.*, 1997], whereas IL-12 and the Th1 cytokines IFN-α and IL-2 seem to play an important role for viral clearance in chronically infected patients [Rossol *et al.*, 1997; Guidotti *et al.*, 1999]. To apply IL-12 genes in gene therapy, a pIL-12 vector was constructed that contained two cytomegalovirus (CMV) promoters to drive the expression of p35 and p40 subunits, respectively. In addition, a pscIL-12 vector was designed with a linker to fuse p35 cDNA with p40 cDNA to producing a single-chain IL-12 protein. The data suggested that the vectors could produce bioactive heterodimeric and single-chain murine IL-12. Furthermore, *in vivo* functional studies also demonstrated that mice co-immunized with a pS vector expressing the major envelope protein of HBV and pIL-12 or pscIL-12 elicited higher levels of IgG2a anti-HBs antibody and of Th1-related cytokine. The success in using a single promoter to express single-chain IL-12 indicated that pscIL-12 should be useful in future applications for gene therapy [Lee *et al.*, 1998].

2.3 HBV Antigen

DNA-mediated immunization has been shown to be an effective way to induce both homoral and cell-mediated immune responses against many different HBV antigens. First, the envelope protein of HBV, i.e. HBsAg, was chosen as a model for DNA vaccination, as it carried the major antigenic determinant of the virus [Mancini *et al.*, 1996; Michel *et al.*, 1995, 2001; Geissler *et al.*, 1998; Davis *et al.*, 1996]. Using the HBsAg transgenic mouse as a model, Mancini *et al.* studied immunization mediated by the S and pre-S2 domains of the gene encoding the HBV envelope protein, and found that the induced immune response resulted in the complete clearance of circulating HBsAg and in the long-term control of transgene expression in hepatocytes. The study showed that T cells were responsible for the down-regulation of HBV mRNA in the liver. This was the first demonstration of potential immunotherapeutic application of DNA-mediated immunization against an infectious disease that raises the possibility of designing more effective ways of treating HBV chronic carriers [Mancini *et al.*, 1996]. Recently, they explored the ability of CpG-containing oligodeoxynucleotides combined with recombinant HBsAg to induce Th1 responses in the same model, and suggested that DNA motifs containing unmethylated CpG dinucleotides within the context of certain flanking sequences enhanced both innate and antigen-specific immune responses, due in part to the enhanced production of Th1-type cytokines [Malanchere-Bres *et al.*, 2001]. In addition, with HBV-transgenic mice, it was demonstrated that the activation of dendritic cells following injection with vaccine containing HBsAg is the vital factor underlying the therapeutic potentiality of vaccine therapy in HBV carrier [Akbar *et al.*, 1999]. As the HBV core antigen (HBcAg) and e antigen (eAg) are highly conserved between HBV subtypes, they are attractive targets for an immune-based therapeutic treatment [Sallberg *et al.*, 1997,1998; Townsend *et al.*, 1997]. Townsend *et al.* showed that intramuscular injections of a novel recombinant retroviral vector expressing an HBcAg-neomycin phosphotransferase II (HBc-NEO) fusion protein induced HBc/eAg-specific antibodies and CD4+ and CD8+ T cell responses in mice and rhesus monkeys [Townsend *et al.*, 1997]. When three chronically infected chimpanzees were immunized with nonreplicating retroviral vector particles expressing the HBc-NEO fusion protein, one exhibited a traditional seroconversion, while the other two showed transient ALT flares and a significant decrease in the serum HBV DNA levels [Sallberg *et al.*, 1998]. Recently, CTL responses against HBV polymerase were assayed. Immunized mice exhibited substantial polymerase-specific CTL responses. This is the first study to demonstrate the generation of a CTL response to HBV pol by immunization. The next task is to investigate the response either in infection models or in transgenic mice that fully replicate HBV. In addition, the validation of HBV polymerase as a target for DNA-based immunization requires further investigation of its potential toxic effects when expressed at high levels in cells [zu *et al.*, 2000].

The expression of cytokine or a costimulatory protein and HBV antigen in the same cells *in vivo* induces stronger cellular and humoral immune response than expression of the antigen alone as demonstrated by several studies of IL-2 [Geissler *et al.*, 1998; Chow *et al.*, 1997], GM-CSF [Geissler *et al.*, 1998], IL-12 [Lee *et al.*, 1998], B7-1 [He *et al.*, 1996], and B7-2 [Zhou *et al.*, 2001]. This could be a novel strategy for the development of therapeutic vaccines against infectious agents. Conventional vaccine combined with CpG oligodeoxynucleotides motifs [Malanchere-Bres *et al.*, 2001], or vaccine with new peptide, which can elicit priming of antigen-specific cytotoxic T lymphocytes [Meng *et al.*, 2001], may also be promising therapeutic approaches and deserve confirmation in further studies. Furthermore, these immunomodulatory agents should be more useful when combined with drugs that are capable of blocking viral replication.

3. DELIVERY METHODS

3.1 Viral Vectors

Delivery of genes for stable gene expression requires the use of an efficient gene delivery system, such as replication defective viral vectors. Currently, mouse retroviral vectors have widely been used in gene therapy, as they provide efficient transduction of a wide range of cell types and the genes are stably integrated and expressed in the host cell. To achieve high transgenic expression in the liver, various murine retoviral long terminal repeats (LTRs) or leader sequences were compared, and higher gene expression was observed by the FMEV-type vector, which contained the spleen focus-forming virus (SFFVp) LTR and the mouse embryonic stem cell virus (MESV) leader, than by the Moloney murine leukemia virus (MoMLV)-based vector [Ohnishi *et al.*, 2002]. To expedite analysis in anti-HBV gene therapy, retroviral vector was used to transfer antisense [Tung and Bowen 1998; Ji *et al.*, 1997]. It is safe to utilize retroviral vector encoding HBcAg to immunize chimpanzees and stimulate immune responses in HBV chronic carrier

chimpanzees; demonstrating retroviral vector immunization may be beneficial in the immuno-gene therapy for chronic HBV infection [Sallberg *et al.*, 1998]. However, conventional retroviral vectors may not be an ideal vector system to deliver genes into hepatocytes *in vivo* because the majority of liver cells are not dividing. Furthermore, retroviral vectors can be generated in only limited quantities and have a broad host-range that does not conduce to the hepatocyte tropism required for HBV therapy. This problem has been overcome by deriving vectors from lentiviruses (a class of retroviruses) that have the ability to infect both dividing and nondividing cells. The lentiviral vectors are derived from human immunodeficiency virus type 1 (HIV-1) [Naldini *et al.*, 1996]. More recently Sung *et al.* have developed a system for producing murine leukemia virus (MLV) pseudotyped with large (L) and small (S) HBsAg for targeting primary human hepatocytes. The MLV (HBV) pseudotype virus remains the strict hepatotropsim of the natural HBV, and does not infect any of the established tissue culture cell lines. The presence of both L and S forms enhanced the surface expression of HBsAg and thus increased virus production. This virus offers a potential liver-specific targeting system for gene therapy [Sung and Lai, 2002].

Gene therapy is not a low risk/benefit approach. Safety is always of paramount importance for clinical gene therapy. Humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses [Xu *et al.*, 2003]. The issues and assays needed to ensure patient safety with this new vector system are still being defined [Podsakoff, 2001]. It has been suggested that the possibility of inadvertent transfer of a mobilized vector to a partner could have potentially serious consequences. Vector mobilization at any level is problematic because it might lead to unwanted recombination events, which could adversely affect a trial subject. More thorough characterization of potential outcomes is necessary before it can be applied in the clinic [Podsakoff, 2001]. The clinical trial of gene therapy for X-linked severe combined immune deficiency (SCID) performed by the French investigators still has been seven of 10 subjects in good health with their immune systems restored by the gene treatment. These findings highlight the potential of gene therapy to correct this otherwise fatal immune disorder without complications, such as graft rejection, that may be seen when hematopoietic stem cells from another donor are used in a "standard" bone marrow transplant approach. However, the leukemia developed in two of 10 infants treated for SCID by gene therapy was observed [Geiger, 2003]. This event is directly related to the retroviral-mediated insertion of the gene products. A key scientific question to be explored is why this problem has only been seen so far in this study of infants treated for XSCID, but not in any of the other clinical trial using retroviral vectors targeted to hematopoietic stem cells or any other trial of gene therapy [Geiger, 2003]. The extensive studies animal models of cancer revealed that genome-wide retroviral insertional tagging of genes involved in cancer in Cdkn2a-deficient mice [Lund *et al.*, 2002] and the new genes involved in cancer were also identified by retroviral tagging [Suzuki *et al.*, 2002]. Therefore, further assessment of the risk to patients must be completed prior to initiation of any new clinical trial.

Adenoviruses (Ad) are hepatotropic when injected intravenously and can be generated at a very high titer, but the use of adenoviral vectors has been limited due to host immune responses against the vector and/or transgene and vector toxicity [Yang *et al.*, 1994]. To decrease side effects associated with viral gene expression, further attenuating of viral gene expression by eliminating viral genes has been attempted. Significantly diminished vector toxicity was obtained in mice treated with E1/E2a/E3/E4-deficient Ad vectors. However, the duration of transgene expression mediated by this vector was reduced [Andrews *et al.*, 2001]. Hodges *et al.* generated Ad vectors with 100K gene deleted, and demonstrated that injection of an [E1-, 100K-]Ad vector *in vivo* is correlated with significantly decreased hepatotoxicity, as well as prolonged vector persistence [Hodges *et al.*, 2001]. These results confirmed that adenoviral vectors with all viral coding sequences deleted offer the prospects of decreased host immune responses to viral proteins, decreased cellular toxicity of viral proteins, and increased capacity to accommodate large regulatory DNA regions [Schiedner *et al.*, 1998]. A similar effect occurs when using an Ad-based vector, the encapsidated adenovirus mini-chromosome (EAM) from which all of the viral genes have been deleted [Kumar-Singh, 1998]. Use of a liver-specific promoter also can reduce immune response to the transgene in adenoviral vectors [Pastore *et al.*, 1999]. The combination of a helper-dependent adenovirus vector and liver-specific promoter resulted in intrahepatic IFN-α expression, which protected the liver in acute hepatitis model [Aurisicchio *et al.*, 2000]. It was reported that episomal segregation of the adenovirus enhancer sequence by conditional genome rearrangement abrogated late viral gene expression. A recombinant adenovirus gene delivery system with the capability of undergoing growth phase-dependent site-specific recombination has been constructed. Because no helper virus is required to propagate these vectors, the problems of recombination with and contamination by helper virus are eliminated [Wang *et al.*, 2000]. Another report showed that inserting inverted repeats (IRs) into the E1 region of the Ad vector could mediate predictable genomic rearrangements, resulting in vector genomes devoid of all viral genes [Steinwaerder *et al.*, 1999].

Adeno-associated virus (AAV), a nonpathogenic, single-stranded DNA virus, can transduce both dividing and nondividing cells, and achieve long-term expression of therapeutic genes with no apparent adverse effects. Most importantly, several groups have documented the ability to deliver sustained liver-targeted transgene expression in an immunocompetent host for more than 1 year, and that the curative level of the gene product from one injection is sustained for long-term in the animal [Wang *et al.*, 2000; wang *et al.*, 1999; Jung *et al.*, 2001; Xu *et al.*, 2001]. Comparable results have not been achieved with any other vector to date. Additionally, a gene 'pill', i.e. an AAV vector administrated perorally, associated with highly efficient and stable gene expression, should render AAV vectors a palatable choice compared with current pharmacological treatment [During *et al.*, 1998; 2000; Xu *et al.*, 2003]. Recently, research has been conducted to reveal the

chromosomal effects of AAV vector integration. It was reported that integrated vector proviruses are associated with chromosomal deletions and other rearrangements [Miller *et al.*, 2002].

Hepatitis B virus with the distinct liver-targeted features is an attractive candidate as a vector for gene therapy of acquired liver diseases. Construction of a vector from HBV DNA has been attempted [Chaisomchit *et al.*, 1997]. Hanafusa *et al.* showed that HBV could carry 63 bp of extra DNA [Hanafusa *et al.*, 1999]. Four novel cir-acting elements were reported to be essential for the viral genome synthesis. According to this result, a recombinant HBV-GFP vector was generated, which can replicate as efficiently as that of the wildtype [Ryu and Lee 2001]. As a first step toward therapeutically useful hepadnavirus vectors, Protzer *et al.* constructed a recombinant DHBV carrying the duck homolog of IFN-α, which efficiently suppressed wild-type virus replication [Protzer *et al.*, 1999]. However, its potential use as a gene transfer system may be limited by its small capacity, due to the small size of the HBV genome.

3.2 Nonviral Vectors

Naked DNA acts as a simple, safe and viable alternative for gene therapy. The use of plasmid vectors expressing the HBV antigens alone, or coexpressing with cytokine, for transfection of muscle fibers has been demonstrated to be a potential immunotherapeutic application against HBV infection [Mancini *et al.*, 1996; Geissler *et al.*, 1998; Chow *et al.*, 1997]. HBsAg-specific humoral or cell-mediated responses are not induced in mice when the muscle-specific human muscle creatine kinase promoter is used in plasmid DNA vaccine. This result suggested use of a tissue-specific promoter that does not drive expression in antigen-presenting cells [Weeratna *et al.*, 2001]. Several approaches aiming at enhancing nonviral transgene delivery have been investigated. One approach is to pulse electrical fields (electroporation or EP) after naked gene injection [Glasspool-Malone *et al.*, 2000]. These advances create new opportunities for nucleic acid vaccine development. Evidence showed that electroporation enhanced that the delivery of plasmid DNA encoding IL-12 to skin [Heller *et al.*, 2001] or to skeletal [Lucas and Heller 2001] muscle. The molecule was efficiently delivered, and one of the molecules that induced (IFN-gamma) was also measured systemically in this successful delivery to skeletal muscle [Lucas *et al.*, 2001]. The use of *in vivo* electroporation in immunotherapy protocols deserves further examination. Another approach is to combine plasmid DNA to other compounds. Poloxamers [Lemieux *et al.*, 2000], aurintricarboxylic acid [Glasspool-Malone *et al.*, 2000], nuclear localization signal (NLS) peptides [Schirmbeck *et al.*, 2001] and some other polymers [Prokop *et al.*, 2002] have been demonstrated to be good candidates for enhancing the efficiency of gene transfer.

When liposome, one of the most popular gene transfer systems *in vitro* in the laboratory [Felgner and Ringold, 1989], was used *in vivo* as liver-directed gene transfer, it was observed that its transfection efficiency depended on its route of administration [Otsuka *et al.*, 2000; Hirano *et al.*, 1998; Mohr *et al.*, 2001], and the efficiency can be increased by liver resection, ischemia or transplantation performed before DNA injection [Otsuka *et al.*, 2000]. Liposome can deliver not only the genes, but also the drugs, to the liver. To prevent degradation of antisense molecules *in vivo*, Soni *et al.* [Soni *et al.*, 1998] showed that liposomes could increase the hepatic delivery and antiviral efficacy of phosphorothioate antisense oligodeoxynucleotides (PS-ODN).

Many attempts for receptor-mediated liver-targeted delivery have been performed successfully. Since asyaloglicoprotein receptor is specific for hepatocytes, DNA-protein complexes using asyaloglicoproteins [Nakazono *et al.*, 1996; Wu and Wu 1992] or protein conjugates, consisting of N-acetyl-glucosamine-modified bovine serum albumin, streptavidin and Poly-L-lysine [Madon and Blum 1996], have been shown to be effective at delivering antisense DNA to suppress HBV gene expression *in vitro*, and this receptor-mediated endocytosis showed no host toxicity [Wu *et al.*, 2001]. An N-glycosylated human IFN-α was generated, which exhibited a significantly higher antiviral effect than conventional IFN-α *in vivo* [Boni *et al.*, 2001]. Complexed with N-acetylglucosamine, a recombinant human adenovirus, which does not naturally infect avian cells, it allowed highly efficient and specific gene transfer into the liver of ducks *in vivo*. This result represents a novel approach to gene therapy for inherited and acquired liver diseases [Thoma *et al.*, 2000]. The functional ability of synthetic galactose polymer ligand was evaluated and poly-(N-p-vinylbenzyl-O-beta- D-galactopyranosyl- [1-4]-D-gluconamide) (PVLA) was found to exhibit higher affinity with hepatocytes than natural ligands [Watanabe *et al.*, 2000]. Oral administration of cholesterol-modified phosphorothioate antisense oligonucleotides (Chol-S-ODNs) has also been shown to target the liver, and has been suggested as a practical method for the long-term treatment of chronic diseases [Okamoto *et al.*, 1999]. In addition, *in vivo* gene delivery to the liver may achieve success by some other nonviral vectors, such as reconstituted remnants of chylomicron, the first nonviral vector to resemble a natural lipoprotein carrier [Hara *et al.*, 1997]. Linear polyethylenmine (IPEI)-mediated transfer was also shown to be a good delivery method in the duck model [Robaczewska *et al.*, 2001].

3.3 Liver Transplantation with Combination Therapy

More recently, a combination of hepatitis B immune globulin (HBIG) and lamivudine has been shown to prevent HBV recurrence effectively in patient post-orthotopic liver transplantation for hepatits B virus infection. Recent studies have revealed that in the combination therapy group no patient redeveloped serum HBsAg or HBV DNA during mean follow-up of 459 and 416 days, respectively. In the monotherapy group, there was a reappearance of HBaAg in the serum of 3 patients (25%) during a mean follow-up of 663 days [Han *et al.*, 2000]. Combination prophylaxis with HBIG and lamivudine is highly effective in preventing recurrent HBV, may protect against the emergence of resistant mutants, and is significantly more cost-effective than HBIG monotherapy with its associated rate of recurrent HBV. Famciclovir and lamivudine also reduced viral replication in patients with recurrent hepatitis B virus infection after orthotopic liver transplantation [Tillmann *et*

al., 1999]. Since the treatment break through is frequent for this specific group of patients, and there are no significant adverse side effects, the use of liver transplantation with combination therapy should be explored.

4. ANIMAL MODELS

The infection of ducks [Mason *et al.*, 1980], woodchucks [Summers *et al.*, 1978], and squirrels [Marion *et al.*, 1980] with their respective animal hepatitis viruses has been an important step in gaining much of our knowledge of HBV infection. The similarity between HBV and the closely related DHBV makes the latter a convenient model for the study of molecular mechanisms of HBV replication and neutralization and for the screening of antiviral agents [Chassot *et al.*, 1993; Mi *et al.*, 1995]. However, DHBV is not typically associated with liver disease. Woodchucks chronically infected with WHV develop progressively severe hepatitis and hepatocellular carcinoma similar to those associated with HBV infection in humans. Chronic WHV carrier woodchucks have become a valuable animal model for preclinical evaluation of anti viral therapy for HBV infection, providing useful pharmacokinetic and pharmacodynamic result in a relevant animal disease model. This model also has significant potential for the preclinical assessment of antiviral drug toxicity [Tennant and Gerin 2001]. However, due to the high level of divergence between HBV and these viruses and the considerable metabolic differences between their hosts and humans, the utility of these models is often limited.

The best animal model to date for infections with human HBV is the chimpanzee. However, because chimpanzees are large-sized and highly intelligent animals, and also an endangered species, their use is reserved for essential experiments.

Mice are not susceptible to HBV infection, but a number of lineages of mice have so far been developed to carry one or more HBV transgenes. Guidotti *et al.* successfully generated transgenic mice that replicated high levels of human hepatitis B virus in clinically important target organs of the liver and kidney. Sera from these mice contain a high titer of viral DNA approaching to that found in the natural chronic human infection [Guidotti *et al.*, 1995]. This model has been demonstrated to be a valuable therapeutic model for HBV [Morrey *et al.*, 1999; Morrey *et al.*, 1999]. However, since the transgenic mouse are tolerant of HBV antigens, there are limitations to the use of transgenic mouse models in the study of the mechanisms by which the anti-HBV cellular immune response leads to liver disease. Recently, an HBV transgenic severe combined immunodeficiency (SCID) mouse was created. These mice consistently supported HBV gene expression and replication. After adoptive transfer of syngeneic, unprimed splenocytes, these mice reproducibly cleared virus markers from the liver and serum, and developed chronic hepatitis. This unique model provided an opportunity to elucidate the pathogenesis of chronic liver disease and to evaluate new approaches aimed at both the virus and the disease [Larkin *et al.*, 1999]. A recent study has provided the first evidence that adenovirus-mediated genome transfer initiated efficient hepatitis B virus replication in cultured liver cells and in the experimental animals from an extrachromosomal template. Allowing the development of small-animal systems of hepatitis B virus infection, and facilitating the study of the pathogenicity of wild type and mutant viruses, virus-host interaction, and new therapeutic approaches [Sprinzl *et al.*, 2001].

An alternative way of developing an HBV-carrying mouse model can be achieved by transplanting human hepatocytes. The hepatitis B virus-trimera mouse was created by the implantation of *ex vivo* HBV-infected liver fragments into lethally irradiated mice, radioprotected with SCID mouse bone marrow cells. Viremia attained a peak between days 18 and 25, while HBV infection is observed in 85% of the transplanted animals 1 month post-implantation [Ilan *et al.*, 1999]. Compared with the trimera model, a newly reported xenotransplant model exhibited some advantages. These mice were susceptible to HBV infection and completion of the viral life cycle. Furthermore, they can be super-infected with HDV. This study demonstrates that human hepatocytes can be engrafted on a long-term basis in mice, and serve as a model for human diseases such as HBV and HDV infection. This model therefore offers an important opportunity of studying multiple aspects of human hepatitis viral infection, and may enhance studies of human liver diseases [Ohashi *et al.*, 2000]. Transplanting human hepatocytes and inoculating HBV generated a model of human hepatitis B infection (HBV) in immunocompetent rats after birth. [Wu *et al.*, 2001]. Other researches have proved that normal human hepatocytes can integrate into the mouse hepatic parenchyma, undergo multiple cell divisions, and remain permissive for a human hepatotropic virus in a xenogenic liver [Dandri *et al.*, 2001].

In addition, the attempts to develop small primate models with hepadnaviruses closely related to HBV have provided a potential animal model for HBV research, e.g., the woolly monkey (*Lagothrix lagotricha*), from which a hepadnavirus (WMHBV) has been isolated [Lanford *et al.*, 1998] and is under investigation [Kock *et al.*, 2001]. The molecular biology of WMHBV should be fully understood before the WMHBV/woolly monkey model system is applied in anti-HBV research.

Better animal models of HBV infections are still extremely needed to test antiviral strategies for eliminating chronic liver disease. It was reported that natural human HBV could enter Wistar rat liver cells through intravenous injection efficiently, and express for a long period [Wang *et al.*, 1996]. This implies the possibility of a rat model infected with HBV.

CONCLUSION

Simultaneously with the increase in our knowledge of HBV molecular biology, especially of the replication mechanisms, a number of virus-specific proteins and nucleotide analogs, such as adfovir, or processes, have also been identified as targets for transgene intervention. Only some of these have been addressed here. Clinical trials indicate that different types of combined therapy may have to be tailor-made for chronic HBV infection. More targets identified, more alternatives there are. Studies in gene therapy for other viruses, e.g. HIV, have been very helpful into anti-HBV research. The effectiveness of combination

genetic therapy has been reported in inhibiting HIV-1 [Strayer *et al.*, 2002; Lisziewicz *et al.*, 2000]. But there are limited delivery systems for synchronous expression of multigenes. Development and testing of combination anti-HBV genetic therapies require both transgenes that effectively inhibit HBV individually or cooperatively, and a vector that delivers these transgenes at high efficiency. Keeping HBV out of the cells and depriving the viruses of infectious ability may be realized by the studies on the early events of the viral life cycle. Although cloning of the DHBV receptor may aid in the identification of the HBV human counterpart [Tong *et al.*, 1999], there are still many unanswered questions in cell culture system. No permanent cell lines are permissive to HBV infection, and primary human hepatocytes are not easily available for *in vitro* infection studies. More importantly, an animal model mimicking natural HBV infection is needed to understand the mechanisms behind the process from acute to chronic states and to optimize the protocols of immunotherapy and/or antiviral therapy. Nevertheless, molecular therapy approaches, because of the promise they show, look set to be increasingly applied in clinical treatment in next few years.

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